

Paramount leadership that failed to deliver freedom

China's economic growth and scientific development bear witness to the towering achievements of Deng Xiaoping. But his death highlights grievous shortcomings that his successor should avoid.

Deng Xiaoping, who died last week, evidently made a great impression on the 1.2 billion citizens over whom he ruled, some of whom will have seen him on his many flesh-pressing walkabouts. To people who met him he was power personified. For China as a whole, he will be remembered fondly as the one who decreed that prosperity is not incompatible with serfdom. It is a short step from there to the belief that serfdom is essential for a booming gross national product.

Since his death, Deng has been widely described as a "pragmatist", meaning that he had the wit to see that China would not hold together unless the lot of its people was improved — and that he was prepared, at least for the time being, to overlook the danger that prosperity might itself engender rebellion. That tolerance vanished when Deng sent in the tanks to Tiananmen Square.

In truth, the misfortune for China and the rest of us is that Deng was too pragmatic by half. He had the courage to endure the Long March as well as the indignities of the Cultural Revolution, but seemingly not the ambition to articulate a role for his new China in the century ahead. If a "paramount leader" has any responsibility at all, it is surely "the vision thing", as US President George Bush once called it.

Deng's successors will be painfully conscious of his failure to leave them a blueprint for the future. They need one now, if only to understand where the past twenty years of breathtaking economic growth are heading. Giving China's private sector access to capital from overseas has predictably stimulated production, a booming export trade and a healthy balance of payments. Deng's formula has also turned hundreds of Chinese into millionaires and whole rural communities into crowds sleeping rough at the city-centre railway stations. The former are congratulated as economic patriots, but in this get-rich-

quick society there is not yet a policy on the growing armies of the impoverished. Compassion is not a Chinese strength.

China's place in the wider world is another conundrum. From the outside it is perceived as an awkward customer, forever raising tension over issues such as Taiwan, arms sales to Pakistan and the like. From the inside, it seems that China is hemmed in by the military might of the United States and what used to be the Soviet Union. If only Deng had thought of a way through this maze, we should all be safer. The best hope is that Deng's successors will be willing to talk about international security more willingly than in the past. The danger lies in China's distinctive blend of chauvinism and timidity.

Modernist and modernizer as he was, Deng's influence on science was good. The old research institutes and, even more notably, the universities have embraced the new competitiveness; the successful among them are on the way to being research conglomerates, with one foot in industry and the other in blue sky. Yet China is not yet making the mark on the international pattern of research that its people's talents, and its declared ambitions, would sustain. That disappointment is one part of the price that China pays for the stultifying political correctness and cronyism of its civic life. Another is the reluctance of thousands of Chinese expatriates to bring scientific skills and experience back home.

Deng was not a modernizer in that regard: personal liberty (of which there is now a lot more in China than there used to be) is fine if it means freedom to move from one place to another or even to buy a ticket for a package tour abroad, but nothing can be allowed to undermine the government's supremacy. If, as seems probable, Deng's successors follow suit, they will have to wait a long time before the harvest of discovery for which they yearn becomes a reality. □

Caught napping by clones

Pleas for ethical advice on mammalian cloning reveal a lack of foresight.

Shortly before this issue of *Nature* went to press, we received an e-mail pleading that a paper, "Viable offspring derived from fetal and adult mammalian cells" (page 810), be removed from it. The paper does what the writer of the e-mail feared: spells out the way in which researchers in Edinburgh extracted the genome of a sheep from a tissue cell, and inserted it into an egg from another sheep, reimplanting it so as to produce a lamb genetically identical to the genome's donor.

The immediate scientific value of this work relates to understanding of the effects of cell differentiation on genomes (see page 769). In the public discussion that followed the premature disclosure of this work by a newspaper last Sunday, researchers were understandably quick to emphasize the technical and legal obstacles to the cloning of humans. But to leave it at that would smack of a psychology of denial under stress. Today's results were a likely consequence of last year's report of cloned sheep from embryos, "as sure as eggs is eggs" as an Eng-

lish cliché so aptly puts it. Cloning humans from adults' tissues is likely to be achievable any time from one to ten years from now. Ethical constraints aside, there are even some rare genetic and medical disorders for which it would be a desirable way for a couple to produce offspring.

The writer of the e-mail urged that the paper be withdrawn from publication pending more thought being given to bioethical issues and access of information. "As the procedure becomes more and more commonplace, its abuse by extralegal or foreign groups is almost inevitable", wrote the Harvard academic. Although publication of this week's paper still leaves substantial technical barriers to be overcome for anyone wishing to adapt it to other mammals, the writer is correct to imply that discussion has been grossly inadequate. At a time when the science policy world is replete with technology foresight exercises, for a US president and other politicians only now to be requesting guidance about what appears in today's *Nature* is shaming. □



Confidentiality of academy panel challenged by environmentalists

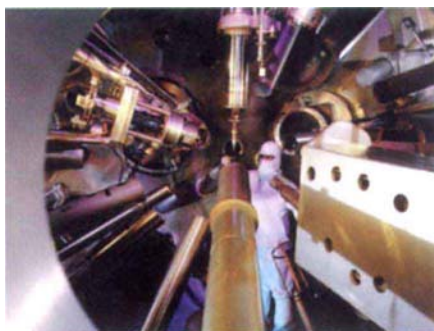
[WASHINGTON] Three US environmental groups have filed suit to block construction of a \$1.1-billion nuclear weapons research facility at Lawrence Livermore National Laboratory in California. They are arguing that a panel of scientists formed to advise the Department of Energy on the project should have carried out its work in public.

The planned National Ignition Facility (NIF) is intended to simulate conditions in nuclear explosions, and its construction has already been given the go-ahead.

But the suit has wider implications, as it highlights growing concerns about the extent to which members of panels set up by the National Academy of Science (NAS) may be under a legal obligation to disclose any advice they provide.

In a lawsuit filed in the Federal District Court for the District of Columbia, the three groups — the Natural Resources Defense Council, Western States Legal Foundation and Tri-Valley Citizens Against a Radioactive Environment — are seeking an injunction prohibiting the department from using any of the technical advice it receives from the Inertial Confinement Fusion (ICF) Committee.

This committee was set up under the auspices of the NAS to prepare a report for the energy department on the scientific and technical readiness of the facility. Its findings



Off beam? Courts may decide whether advice on laser research (above) should be made public.

are expected to be made public next month.

The environmental groups claim that the ICF committee should be subject to the Federal Advisory Committee Act, which requires such panels to operate in public. The suit also claims that the panel's membership violates the act because it is unfairly balanced and includes members with inappropriate special interests.

A spokeswoman for the energy department says that it had not reviewed the complaint, and declines to comment. But William Colglazier, executive officer of NAS and its subsidiary, the National Research Council, says the academy maintains that the act does not apply to its committees —

despite a January ruling by a federal appeals court in a similar but unrelated case.

The case before the appeals court involved a panel formed by the NRC for the National Institutes of Health to revise guidelines on the care and use of laboratory animals. Animal rights organizations sued on the grounds that the panel should have followed the act's provisions.

If upheld, the appeals court ruling could force the academy to begin complying with the act in those instances, such as the NIF panel, where non-government committees are used by government agencies. The academy is appealing against the ruling. Until the appeal is completed, Colglazier says, the energy department and NAS have agreed to continue the operations of the ICF panel unchanged.

The issue of the committee's membership has been simmering for some time. Thomas Cochran, director of the Natural Resources Defense Council's nuclear programme, complained to Bruce Alberts, chairman of NRC, in a letter last December that "without question the current membership of the NRC's ICF committee is not balanced" and "is in clear violation of the NRC's policy" designed to ensure that committee reports are free from conflicts of interest or bias.

Ten members of the committee either have ties to the energy department's fusion programme or to Lawrence Livermore "or have already voiced very positive views regarding NIF," says Cochran. Several, including Steven Koonin, provost of California Institute of Technology and chairman of the panel, have served on earlier reviews of the ICF programme.

In response, Colglazier says that the panel's composition reflects its narrow charge of determining whether there may be outstanding scientific and technical issues that should be resolved before starting to build NIF. The energy department had already reviewed and resolved the larger issue of whether NIF should be built, he says.

Colglazier says the NRC followed its normal procedures in picking members of the committee, with about half the members having "intimate knowledge" of ICF or the NIF project, and the rest chosen from other scientific disciplines who might bring a "fresh perspective".

Howard Crystal, an attorney representing the three environmental groups, said last week that District Judge Paul Friedman, who has been assigned to consider the suit, has not yet indicated when the case is likely to be dealt with.

David Kramer

US decision 'will not limit gene patents'

[LONDON] The US Patent and Trademark Office has confirmed an informal announcement by one of its officials last week that it has decided to grant patents for expressed sequence tags (ESTs), the short sequences of DNA that uniquely identify whole genes expressed in cells (see *Nature* 385, 670; 1996).

But the patent office says that, although any such patent would be based on the novelty and utility of ESTs as probes for the corresponding full-length gene — for example in forensic tests — it would not cover any further functions or uses of the gene. Many researchers in the field do not want the patents to cover such further functions.

Lawrence Goffney, deputy commissioner of patents and trademarks, says the decision means that any functions subsequently discovered for the gene whose EST is patented would be separately patentable, as long as they were not already obvious from the EST.

ESTs can be mass-produced from samples of messenger RNA, providing a quick way to

log the thousands of genes being expressed in a cell at a particular time. Because it is principally commercial companies that have the high-throughput DNA-sequencing capabilities to find large numbers of ESTs, scientists have expressed concern at the idea that patents could be granted for ESTs in case these precluded further research on the corresponding genes.

As a result of the new decision, patent owners would be able to license other researchers to use the ESTs, but this would only cover research on the whole gene in rare cases where the EST encompasses the entire gene sequence.

John Doll, director of the biotechnology patent examining group at the patent office, says that the decision to grant patents for ESTs with demonstrated utilities as probes was made at the end of last year. No such patents have been granted, although the procedure has been initiated in a handful of cases. "We have approximately 350 [patent] applications that are claiming over 500,000 ESTs," says Doll.

Claire O'Brien

Marijuana research gets backing at NIH

[WASHINGTON] A panel of scientists put together by the National Institutes of Health (NIH) said last week that the potential therapeutic uses of marijuana deserve further research and that — based on what they admitted to be scant data — the smoked drug is probably effective in some conditions.

"For at least some indications, there is a rationale for looking further into the therapeutic effects of marijuana," said William Beaver, a professor of pharmacology at Georgetown University in Washington DC, who chaired the panel.

The findings will add heat to the debate prompted by referendums last autumn in Arizona and California in which voters chose to legalize doctor-sanctioned smoking of marijuana for medicinal purposes. The US government has since threatened to prosecute doctors who prescribe the drug, a move that the *New England Journal of Medicine* has described as "misguided, heavy-handed and inhumane".

Separately, the Federation of American Scientists last month urged President Bill Clinton to instruct the NIH to carry out marijuana research, arguing that such research had been neglected "primarily for political reasons".

Last week's two-day workshop involved ten NIH institutes and divisions, and was led by the National Institute on Drug Abuse (NIDA). In a report to be completed next

month, the eight-member panel that led the workshop will make formal recommendations on whether research on therapy using smoked marijuana should be pursued.

The panel's preliminary comments at a press conference at the end of the workshop were based on presentations by speakers who had reviewed hundreds of papers, mainly on the therapeutic value and clinical pharmacology of smoked marijuana and its major active ingredient, Δ -9-tetrahydrocannabinol (THC). "It looks promising enough to recommend that there would be some new controlled studies," said Beaver. "Which ones? We're going to have to discuss this further."

At present the NIH — the only legal source of marijuana for experimental purposes — does not fund any studies on the therapeutic use of marijuana, officials said. But last year NIDA, which supplies NIH institutes with the drug, provided marijuana for three studies on its metabolic impact and deleterious effects.

NIDA director Alan Leshner stressed NIH's "openness and willingness" to consider proposals from extramural investigators on marijuana as therapy. He said that the institutes had received just one such protocol in the past decade. It was rejected on scientific grounds. The study, proposed last year, would have examined marijuana's use in people with weight loss from AIDS.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Herbal remedy? *Cannabis indica* in growth.

Beaver stressed that there is a lack of data on smoked marijuana as a therapeutic agent, although THC is a legally prescribed drug for nausea and vomiting in cancer chemotherapy and for appetite stimulation in wasting diseases including AIDS. Controlled studies of smoked marijuana face obstacles that include the difficulty of creating an effective placebo, the need to administer the drug at short intervals, and its long-term side-effects.

Meredith Wadman

High-level ethics committee 'needed to guide genetics policy'

[WASHINGTON] A panel of advisers to the US National Human Genome Research Institute (NHGRI) last week unanimously endorsed a proposal to create a high-profile committee to help guide administration policy on the ethical, legal and social implications of genome research.

The committee would operate in the office of the Secretary of Health and Human Services (HHS). It would take over at a higher level the role of the group that at present helps to shape policy on such issues within the institute, formerly the National Centre for Human Genome Research.

The reorganization was endorsed last Friday (21 February) by the National Advisory Council for Human Genome Research. It was proposed in a report prepared by an evaluation committee that was asked last spring by Francis Collins, director of the institute, to assess the role of the joint National Institutes of Health (NIH)/Department of Energy Working Group on the Ethical, Legal and Social Implications of Human Genome Research — known as the ELSI working group.

The future of the working group had become controversial after Collins had said that budget cuts would mean that it would be

able to meet only once a year (see *Nature* 381, 357; 1996). Working-group members also complained that the genome research centre was muzzling it on controversial topics, and threatening its autonomy by establishing a separate Office of Policy Coordination.

The evaluation committee reported last December that the working group's mandate was "inadequately defined" and "much too broad" to be satisfied by a single body and that its low status as a subcommittee within the two departments "is not commensurate with the more global role of some important policy formulation".

The committee made three recommendations. First, the working group should be restructured as a 12-member ELSI Research Evaluation Committee, to advise Collins and his counterpart in the energy department on the quality, scope, direction and outcome of extramural ELSI research funded by NHGRI and the department.

Second, it urged that NIH director Harold Varmus should coordinate information and direction on ELSI projects within all 24 NIH institutes, divisions and centres, to avoid duplication and to standardize research protocols.

Third, the committee proposed that

responsibility for advising on and shaping government policy on ELSI issues should be shifted to a new independent Advisory Committee on Genetics and Public Policy in the office of the HHS secretary. This would provide a public forum for policy discussions on current and emerging ELSI issues, from health-insurance discrimination to the quality of genetic services.

Some members of the advisory council expressed concern about a proliferation of committees. Kurt Hirschhorn, a liaison member, said that he spoke for both the American Society of Human Genetics and the American College of Medical Genetics in stating "one real concern" about the proposed changes: the overlap of the HHS-level Advisory Committee on Genetics and Public Policy with existing groups such as the National Bioethics Advisory Commission. He called instead for "a single bioethics national effort".

But at the end of last week's meeting the council endorsed the report's recommendations, on condition that NHGRI staff would work out details of the shape and function of the ELSI Research Evaluation Committee and present them to the council at its next meeting in May.

M.W.

Cloning technique 'reveals legal loophole'

[LONDON] Concerns over a possible loophole in UK law that could allow the cloning of humans have been stimulated by the publication this week of research results describing the work of scientists in Scotland who have successfully cloned a lamb from cells of an adult sheep (see page 810).

Some claim the technique, which could in theory be used to clone humans, may not be covered by the Human Fertilization and Embryology Act of 1990, which is intended to outlaw human cloning, as it uses cells taken from adult organisms.

"My view is that the legislation is restricted to [prohibiting] the cloning of embryos," says Sheila McLean, professor of law and ethics in medicine at the University of Glasgow. "But this technique is not about embryos, it is about adults."

The 1990 act states that an embryo cannot be created outside the human body without authorization. It says permission will not be given to "replacing a nucleus of a cell of an embryo with a nucleus taken from a cell of any person, embryo, or subsequent development of an embryo".

David Shapiro, executive secretary of the London-based Nuffield Council for Bioethics, argues that any possible weaknesses in the law should be eliminated as soon as possible. "If there are legal doubts, the matter should be resolved quickly and legislation brought in."

This view is echoed by Baroness Mary Warnock, who chaired the government advisory committee on human fertilization and embryology whose report, published in 1984, formed the basis of the 1990 act.

Warnock says that the act could only include research that was current at that time. "We didn't know about the possibility of cloning from adult cells, and the act probably now needs to be amended to outlaw all human cloning."

The research has been carried out by scientists from the Roslin Institute in Edinburgh, part of the Biotechnology and Biological Sciences Research Council, and PPL Therapeutics, an Edinburgh-based biotechnology company which conducts research into breeding animals for medicinal products such as human protein.

The findings were prematurely disclosed by a British newspaper last weekend. Following publication of the news, the value of PPL's shares rose 25 pence to around £3.60 (US\$5.80) on Monday morning.

The research builds on a paper published last year (see *Nature* 380, 64-66; 1996) reporting on work by the same researchers in which they successfully cloned lambs from the cells of embryos. This time, however, they used the nucleus of cells from an adult sheep and placed it in an unfertilized egg which had its nucleus removed. This was then artificially

inseminated into a surrogate sheep. A lamb, named Dolly, which is genetically identical to the donor sheep, was born a few weeks ago.

A spokesman for PPL dismissed fears on Monday that the technology could lead to the cloning of humans, partly because scientists agree that such a step would be unethical. But ethical and religious organizations have voiced concern at the potential implications.

Nicholas Coote, assistant general secretary of the Roman Catholic Bishops Conference of England and Wales, says that every human being has the right to two biological parents. At the same time he disagrees with those who suggest that the ability to self-reproduce contradicts the Church's teachings that individual creation is unique. "If I have a clone of me, I am still unique as my clone has a consciousness that is not mine."

Jeremy Rifkin, president of the Foundation on Economic Trends in Washington DC, is heading a worldwide coalition of 300 religious and ethics organizations which responded to the news of the Edinburgh work by demanding a worldwide ban on human cloning, suggesting that it should carry a penalty "on a par with rape, child abuse and murder".

Representatives of other church organizations have also voiced concerns. However, they differ over the extent to which cloning should be used, even in animals. Martin

Robra, executive secretary of the World Council of Churches, says there should be a moratorium on research and application of cloning techniques until ethical questions are satisfactorily resolved.

But Mary Seller, a member of the Church of England's Board of Social Responsibility, says that "the antics of a few cranks and Hitler types" should not interfere with such research. "Cloning, like all science, must be used responsibly. Cloning humans is not desirable. But cloning sheep has its uses," says Seller, who is reader in developmental genetics at the United Medical and Dental School of Guy's and St Thomas's Hospitals in London.

Ehsan Masood



Japan decides to burn plutonium stocks

[TOKYO] Faced with a series of setbacks to its fast-breeder reactor programme, Japan has decided to push ahead with proposals to burn its growing stockpile of plutonium in conventional light-water nuclear reactors.

Last week, the Federation of Electric Power Companies approved a plan to use mixed plutonium-uranium fuel (MOX) in conventional reactors. Under the plan, MOX will be used in four reactors belonging to Tokyo Electric Power Company and Kansai Electric Power Company from 1999.

By 2010, the practice will have spread to between 16 and 18 reactors operated by 11 utility companies. The federation's move follows a decision earlier this month by the government to accept a recommendation from the Atomic Energy Commission to push forward with what is called the "plutothermal" programme.

The hands of both the government and industry have been forced by serious setbacks in the development of fast-breeder reactors. Technical difficulties meant that the government had repeatedly to delay the forecast start of commercial fast-breeder reactors from a date of 2010 set in the early 1980s to the present estimate of around 2030.

But even that now seems optimistic following a sodium leak at the Monju fast-breeder reactor that has stopped the fast-breeder programme in its tracks. Some industry observers believe that Japan will never have commercial breeder reactors because industry now sees them as commercially nonviable.

This leaves the serious problem of dealing with Japan's growing stockpile of plutonium derived from the reprocessing of spent nuclear fuel, which could in theory be diverted to produce nuclear weapons.

The government and electricity industry still face a major hurdle in meeting the proposed schedule for introduction of MOX fuel. The governors of Fukui, Niigata and Fukushima prefectures, where two-thirds of Japan's nuclear reactors are located, have both told the central government in Tokyo that they will not license reactors to burn MOX until the Monju accident is thoroughly investigated.

The government released a report on Monju last week confirming that the sodium leak was caused by the fracture of a thermal probe in the sodium cooling system (see *Nature* 379, 196; 1996).

David Swinbanks

Abortion foe blamed for research head upset in Australia

[SYDNEY] The Australian cabinet has become embroiled in controversy over allegations that a legislator with strong anti-abortion views may have helped to block the appointment of a leading candidate as chairman of the National Health and Medical Research Council (NHMRC).

Many in the medical research community have expressed anger that the post was not awarded to John Funder, a leading endocrinologist and the first nominee of the health minister, Michael Wooldridge. The position is to be taken instead by Richard Larkins, a widely respected physician at the University of Melbourne.

It is widely believed that the candidacy of Funder, who is director of the Baker Medical Research Institute in Melbourne, was undermined by Brian Harradine, one of two independents holding the balance of power in the Australian Senate.

Harradine, a conservative Roman Catholic, is a critic of abortion under the national health scheme. He is also an opponent of research into birth control, and of the so-called 'abortion pill', RU486, imports of which the government has agreed to ban at his urging. Harradine has criticized Funder — also a Catholic — for his more liberal views on these matters.

Although Funder had received a draft contract from Wooldridge's department, it was subject to cabinet approval. When this was not announced following a scheduled meeting of the cabinet, the opposition released a petition from 24 senior researchers who urged the government not to succumb to "external political factors" based on Funder's "perceived beliefs on sensitive bioethical issues".

Harradine denies he has influenced the cabinet's decision not to appoint Funder or that his religious and ethical views have been factors. But he acknowledges that he made unspecified "representations" on behalf of an unnamed voter.

Despite a pre-election commitment last year, the government has shown no enthusiasm for boosting the NHMRC's current budget of A\$150 million (US\$116 million) for peer-reviewed grants. But Larkins, showing his independence of the conservative government's agenda, seized the opportunity of his appointment to state his belief that biomedical research requires a sustained increase in public funding.

Peter Pockley



Wooldridge: lacked cabinet backing.

Holland picks 'superleague' plan, despite criticisms

[PARIS] The Netherlands, ranked tenth in the world in terms of scientific output, is embarking on a reform of its research system that could lead to a 'superleague' of ten university research schools. A shake-up of NWO — the Dutch equivalent of the US National Science Foundation — and the creation of a system similar to Germany's Max Planck Institutes are also on the cards.

Under the proposed 'superleague' scheme, up to ten research schools would be chosen by NWO as centres of international excellence, on the basis of their past performance and future plans submitted in response to a call for proposals due to be issued later this year.

The superleague schools would be reserved half of the DFL200-million (US\$106-million) annual research funds provided for the country's 100 or so research schools. The research schools were set up five years ago to sharpen the focus of research at the Netherlands' 13 research universities by regrouping teams in particular disciplines.

Another reform targets NWO itself. This is at present made up of three layers. Individual research councils — such as the Fundamental Research on Matter Agency (FOM), a body roughly equivalent to Britain's former Science and Engineering Research Council — answer to an umbrella body, the Research Council for Natural Sciences, which reports to the NWO general board.

One proposal being considered would break up this umbrella body into a series of research councils for individual disciplines reporting direct to the general board. Under this plan, FOM would become the physics research council.

At present, both NWO and the Royal Netherlands Academy of Science also manage their own research institutes. But the science minister, J. M. M. Ritzen, is keen that they should cease to do so, arguing that this conflicts with their responsibility for funding university research.

The two organizations are scheduled to submit a final proposal to the government next month. In one scenario, their institutes would be combined into a system similar to the Max Planck Institutes in Germany, and would be run by a board supervised jointly by NWO and the academy.

One result could be that FOM would lose direct control of its institutes, such as those for nuclear research, nuclear fusion and free-electron lasers. But some researchers oppose such a move. Ronald Griessen, a Swiss national who works on superconductivity at the Free University of Amsterdam, argues that it would undermine FOM's centralized

system of evaluation.

In general, Dutch scientists have broadly welcomed the goals of the reforms, namely to concentrate resources, increase competition between researchers and boost the visibility of Dutch research abroad.

But many remain sceptical that these goals will be achieved by the proposed changes, which are the latest in a series of reforms by successive governments. Scientists are concerned in particular that the superleague proposal may in fact harm research.

Critics claim that selection at school level is inappropriate, arguing that this is best carried out at the level of individual groups. They also question the feasibility of comparing schools in different disciplines. They say that schools excluded from the top ten will have to compete for the remaining half of the research funds, and that this will penalize good groups at these schools and hinder the development of young universities and emerging schools.

NWO and the academy are scheduled to submit a detailed proposal to the government next month on how the superleague should operate. They are expected to recommend asking schools to submit grant applications from a few select groups rather than from the school as a whole. The winning consortia will each receive between DFL3 million and DFL10 million a year over ten years, with a review halfway through this period.

The fact that NWO — rather than the universities — will be responsible for selecting the winning schools reflects a desire to give the organization a greater say in the distribution of the universities' DFL2.6-billion research funds. Although research councils in most countries distribute 25 to 30 per cent of the budgets for university research, the Netherlands distributes only 12 per cent in this way.

Most Dutch scientists seem to agree that NWO should be given a greater say in national research spending. They argue that, with its well developed system for assessing grant proposals and its broad view of science, it is better placed than individual universities to promote open competition and coherent research strategies at national level.

Indeed some people, such as George Robillard, director of the Groningen Biomolecular Science and Biotechnology Institute, say that the DFL100 million earmarked for the superleague might be better spent by simply transferring it to NWO, where the money could be used to expand an existing scheme — similar to the US system of Howard Hughes professorships — under which leading groups are awarded large grants for five-year periods.

Declan Butler

Basic research gets bipartisan treatment

[WASHINGTON] A bipartisan group of US senators has set up a Science and Technology Caucus which, they promise, will work to build political support for basic research and for partnerships between government and industry.

The centrist group has been founded by Joseph Lieberman (Democrat, Connecticut) and Bill Frist (Republican, Tennessee). Frist, a heart surgeon by profession, is the new chairman of two Senate subcommittees which between them oversee most non-military research and development carried out by the government.

The first meeting of the caucus, two weeks ago, brought a dozen prominent research administrators and industrialists to talk to senators about the government's role in 'fostering' technology. It was co-hosted by Frist, Lieberman, Pete Domenici (Republican, New Mexico) and Jay Rockefeller (Democrat, West Virginia).

Frist says the meeting was a great success. "The important thing is that all the senators stayed for the whole three hours," he says; this "says a lot about the importance they attach to the issue". According to Frist, the caucus is likely to be expanded, with the rest of the 100 senators invited to join. "We want to focus on the long-term role of the federal government in research and development," he says.

Speakers at the caucus included Paul Horn, research vice-president of IBM, and Robert Martin, chief technical officer of Lucent Technologies, as well as officials in the Clinton administration. Horn and Martin gave strong backing to the role of government in supporting technology programmes and basic research.

In contrast, Raymond Gilmartin, chairman of Merck, supported a clear division of



Frist: support at the heart of Congress.

roles between government and industry, with the former sticking to basic research and creating a "free-market business environment" for the industry to get on with its own drug development work.

Frist, who was elected to the Senate two years ago, says he is the first practising physician to enter the Senate since 1928. As director of the heart and lung transplant programme at Vanderbilt University in Nashville, Tennessee, he published more than a hundred research articles, and wrote a book, *Transplant*, on the social and ethical implications of organ transplants.

The 45-year-old senator was named last

month as chair of the science, technology and space subcommittee of the Senate commerce committee, which oversees the space agency NASA and the National Science Foundation. He also took the chair of the newly created public health and safety subcommittee of the Senate labor committee, which has jurisdiction over the National Institutes of Health (NIH) and the Food and Drug Administration. Together with his place on the powerful budget committee, these posts indicate that Frist will emerge as a pivotal figure in US research policy.

"There is huge support among senators for continued and further investment in research and development," says Frist. He says he would like the NIH to get increased funding of "about 5 per cent" this year, against the 2.6 per cent proposed by President Bill Clinton.

The formation of the caucus is seen in Washington as further evidence that the government is edging back towards bipartisan support for science and technology, after two years of bitter arguments, especially over technology policy. Republicans in the Senate have recently voiced strong support for science and technology spending, and the Congress is unlikely to seek cuts in the Clinton administration's proposed budget for science and technology.

Colin Macilwain

Budget 'windfall' may tempt Canadian researchers back home

[OTTAWA] The Canadian government plans to use money made unexpectedly available as a result of its tight fiscal policy to set up a foundation to improve research facilities, hoping this will help to attract back Canadian researchers working overseas.

The creation of a Canada Foundation for Innovation is one of the few new initiatives to be included in the federal budget announced in Ottawa last week. The foundation will receive about C\$180 million (US\$133 million) a year for five years from the government, and is intended to raise a slightly higher figure from private sources.

The money will be used to modernize the research infrastructure at universities and research hospitals in the areas of science, health, engineering and the environment. The foundation will be chaired by John Evans, formerly president of the University of Toronto, founding dean of McMaster University medical faculty, and director of the World Bank's population, health and nutrition department.

Evans, who is currently chairman and chief executive of Allelix, a biotechnology research company, says that many bright Canadians go abroad for postgraduate work and stay there, despite their wish to return,

because of the lack of adequate facilities in Canada. The foundation will play an important role in encouraging them to "come back and perform at a very high level".

Plans for the foundation have been widely welcomed. Louis Siminovitch, director emeritus for research at Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, calls it "an excellent idea". But he emphasizes that the foundation needs to address the problem of operating funds for the laboratories. "We'll have equipment and infrastructure, but our capacity to use it will be severely limited," says Siminovitch.

The situation is certainly serious. The Council for Health Research in Canada, which before the budget lobbied the government heavily for an overall 10 per cent increase in research funds, points out that funding for university research has declined by 14 per cent over two years. Taking inflation into account, the drop is even greater, at 25 per cent. Between 1994 and 1996, funds for the three federal grants councils dropped from C\$830 million to C\$782 million.

Paul Martin, the Liberal government's finance minister, has been applying policies that include strenuous deficit-cutting measures. These have brought the

national deficit to less than C\$19 billion, its lowest for many years. As this is more than C\$5 billion below his target, Martin is left with a windfall, about a quarter of which will go to the new foundation.

The innovation foundation will be an independent corporation, at arm's length from government. A board selected from the private sector, researchers and academics will provide grants to institutions to develop computer networks and databases, to buy equipment or to build research centres.

Those applying for the grants must agree to raise 60 per cent of the total they require from the private sector or from provincial governments. The federal government estimates this will bring up to C\$2 billion into the scheme.

The government has also announced that the Canadian Networks of Centres of Excellence will be made permanent, with an annual commitment of C\$47.4 million.

The National Research Council's Industrial Research Assistance Program, which provides advice from experienced scientists to small and medium-sized enterprises, will be maintained at C\$96.5 million a year.

David Spurgeon

Russian rocket offer holds key to Cluster relaunch

[MUNICH] Plans for a relaunch of the European Space Agency (ESA)'s Cluster mission, which was destroyed last summer when its Ariane-5 rocket exploded, now appear to hinge on an offer from the launch company Arianespace to arrange a cheap launch on a Russian rocket.

The mission had been designed to use four satellites to study the solar wind and the Earth's magnetosphere. Shortly before a meeting of ESA's science programme committee last week, it seemed likely that the agency and its member states would be unable to agree a deal for a relaunch. The agency had been unable to persuade Arianespace to provide a cheap launch on the grounds of the company's responsibility for the Ariane-5 explosion. And the three most crucial member states — Britain, France and Germany — had said they were unable to pay for replacement scientific instruments.

But, only a few hours before last week's meeting, Arianespace presented a new offer, to use two Russian Soyuz launchers rather than Arianes. That would bring ESA's share of the mission's costs within the agreed limit of ECU210 million (US\$184 million) for the satellites and launch.

The ESA committee agreed to give Arianespace and the space agency one month to study the costs and feasibility of the offer. In a reversal of normal practice, it also asked the agency to try to reduce costs further so that the ECU210 million would also pay for the scientific instruments.

If this rather optimistic proposal fails, the mission

could still go ahead — but much later. Cluster's spare satellite, Phoenix, would be launched in any case at the end of this year to carry out part of the mission's scientific programme. The other part of the mission's science — three-dimensional measurements of the magnetosphere — could be revived with the launch of four newly designed mini-satellites. This depends on a full scientific and financial assessment, which ESA is to finance. The assessment will take about a year.

At last week's meeting, the science programme committee was also asked to take note of the space science advisory committee's draft proposals to delay missions in the long-term space science plan, Horizons 2000, to cope with the large budget cuts ESA is facing.

But member states, as represented by their delegates to the committee, were divided in their response. Paul Murdin from Britain's Particle Physics and Astronomy Research Council describes the proposed delays as "unacceptable". He says the advisory committee should adopt a more positive attitude to the budget cuts by investigating "faster, cheaper, better" options, to use the words of NASA, the US space agency.

Lodewijk Woltjer, chairman of the advisory committee and an associate of the Observatoire de Haute Provence in southern France, defends the committee's position. "All we did was add up the numbers, and they added up to trouble," he says. "If Europe doesn't want to pay for a proper space science programme, then we have no choice but to delay missions." But he adds: "If people want to fantasize otherwise, we can't stop them."

Alison Abbott



Germany may relax animal experiment rules

[MUNICH] The German cabinet last week agreed changes in legislation on animal welfare which, if approved by parliament, could make life easier for German biologists by reducing bureaucratic requirements.

German laws controlling the use of animals in research are the strictest in Europe. One suggestion agreed by the cabinet is that research protocols using animals that are not approved within three months of submission to regional government offices should be taken as approved; in some regions, the process can at present take up to six months.

Another proposal is that small changes to a protocol required during the course of a research project need be notified only to the authorities, rather than having to go through the full approval procedure.

In contrast to the current rules, which require animals to be killed after each experiment, it would be possible to use an animal for several experiments, provided it is killed after the last one. And a requirement that only qualified veterinary surgeons or zoologists should be permitted to operate on animals will be relaxed, allowing foreign scientists qualified to operate in their home countries to do so in Germany.

Not all the proposed changes would reduce the burden of regulation. For example, the bill containing the proposals says that animal experiments carried out for teaching purposes would have to be supervised continuously by a qualified veterinary surgeon or zoologist, rather than being left partly to technical assistants.

A.A.

Abbott sues Chiron over 'secrets' in heads of recruits

[SAN FRANCISCO] In a new and unusual interpretation of the law on trade secrets, the US pharmaceutical company Abbott Laboratories is suing its smaller biotechnology competitor, Chiron, claiming that it has raided the pharmaceutical giant's staff to obtain confidential information.

The complaint, filed in the US District Court in Chicago, alleges that 15 high-level scientific and marketing managers recruited from Abbott will inevitably disclose specialized secret knowledge — whether or not they have done so already — and that Chiron and its subsidiary, Chiron Diagnostics, are liable.

The suit claims that over the past three years Chiron and its subsidiary systematically identified and hired individuals with special knowledge about Abbott's immunoassays for HIV, the hepatitis-B and C viruses, human T-cell leukaemia virus, Chagas disease and cancer. It asks for \$75,000 in damages and an injunction to prevent Chiron from recruiting Abbott employees for a certain period. After that date, Chiron would be able to hire only a limited number of people from its competitor.

Chiron's spokesman, Larry Kurtz, says his company will vigorously oppose the suit. "We don't feel we or any of our people have done anything wrong," he says, adding that Chiron is very cautious about proprietary information from external sources, as it could affect the company's own patent status. Kurtz says that Chiron requires all new employees to sign agreements that acknowledge the confidentiality of information from other companies, as well as from within Chiron.

Abbott claims that Chiron intentionally hired the employees to obtain trade secrets and proprietary information about technology and processes.

Some legal experts say that companies are increasingly likely to apply the theory of "inevitable disclosure" to competitors that lure away staff with knowledge of company technology, research and marketing.

Judy Brown, a specialist recruitment consultant, argues that recruiters look primarily for technical skills and compatible backgrounds in research staff, not trade secrets.

But Julie J. Furer, an attorney for Sanchez & Daniels in Chicago who is representing Abbott, says the Abbott case could give recruiters pause for thought when targeting high-level scientists from competing companies. Her firm's arguments are based on similar suits by FMC Corporation, Campbell Soup Company and PepsiCo. PepsiCo successfully used the argument in 1994 to prevent William E. Redmond Jr from going to work for its rival Quaker Oats.

Sally Lehrman

Culture clash tarnishes the image of star laboratory

[MUNICH] A bitter feud between scientists and administrators is threatening to destroy the promising scientific reputation of a recently created research institute in the former East Germany that has been held up as a star of the country's restructured scientific landscape.

Problems at the Institute for Molecular Biology (IMB) in Jena, in the state of Thüringen, came to a head earlier this month with the resignation of the only scientist on its decision-making supervisory board, Manfred Eigen, the Nobel prizewinner and emeritus director of the Max Planck Institute for Biophysical Chemistry in Göttingen.

Following last year's resignation of Lothar Späth, head of the Jena-based optical company Carl-Zeiss, the supervisory board, or 'Kuratorium', now comprises only officials from the federal and state governments, which provide the IMB's DM25-million (US\$14.8-million) annual budget.

In his letter of resignation, Eigen argues that the scientific activities of the institute, which he helped to set up in 1991, have been compromised by weak scientific leadership and an inappropriate management structure which gives too much power to the administrative director.

Earlier this year, Eigen demanded a change in the institute's structure to correct these problems, as well as the resignations of the scientific director, Stefan Diekmann, and the administrative director. But he received no response, and he complains in his letter that he has received no official support for his efforts to protect the IMB from "scientific insignificance".

Eigen is the latest in a list of scientists who have abandoned their association with the institute's management. Others include Peter Schuster, head of the IMB's evolutionary biology research group and former scientific director, and Rudolf Rigler, professor of medical physics at the Karolinska Institute in Stockholm, Sweden, who was head of IMB's scientific council, the Wissenschaftlicher Beirat.

The IMB was founded after Germany's science council, the Wissenschaftsrat, had recommended the research institute on which it is based as one of the few institutes of the former East Germany's Academy of Science whose activities should be continued after reunification. Eigen supported the appointment of Schuster, an Austrian evolu-

tionary biologist and one of his former students, as scientific director.

The institute was able to attract top-class scientists from west Germany and outside to take over key posts. But problems started after Schuster, frustrated at the bureaucratic difficulties he faced, resigned as scientific director in 1994 to go back to full-time research.

Schuster had tried to replace the leadership structure of the IMB, in which the administrative and scientific director share equal decision-making authority, with one that would have strengthened the influence of department heads. But his supporters say that his efforts were blocked by opposition from government officials.

After Schuster's resignation, complaints began to grow among the institute's 130 scientists about a repressive administrative regime and lack of support from the new scientific director. Scientists claim that their applications for research grants are needlessly delayed or held back by the administration. They also complain that the directors delayed releasing money received from research agencies, and about numerous trivial administrative requirements which distract them from their scientific work.

The difficulties are said to have been fanned by personal quarrels within the institute, and researchers' claims that a lack of open discussion has increased the atmosphere of mutual suspicion. For example, regular evaluation reports from the Wissenschaftlicher Beirat are passed to the Kuratorium but kept secret from the scientists involved.

A further source of conflict has been the issue of cooperation with industry, particularly EVOTEC, a Hamburg-based biotechnology company co-founded by Eigen, who, along with Rigler and Schuster, remain partners. Eigen claims that collaboration between EVOTEC and IMB has been deliberately hindered by the institute's directors — a charge the directors deny.

The non-German staff at the IMB, such as Schuster, appear to have suffered most from the problems. Last year the contracts of two of his four group leaders were not renewed — even though they were directing several large research projects with years to run.

The two scientists appealed to the Kuratorium and made their complaints public, both offences against official procedures, for which they were banned from IMB premises. As a result, they had to arrange to meet their PhD students privately.

Their departure — which Schuster says was "prompted by personal animosities not by scientific considerations" — caused a breakdown of relations throughout the insti-

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Old meets new: traditional habits linger in Jena, an ancient town in former East Germany.

tute, and was one of the factors that led to Schuster's resignation as scientific director.

According to Diekmann, the IMB did not want to "reinforce the structure of Schuster's department" before a planned evaluation was carried out by the Wissenschaftlicher Beirat. Ironically, the loss of the two group leaders caused such disruption within the department that this evaluation has never been carried out. Schuster is now negotiating to relocate his entire group elsewhere in Germany.

Ministry officials have up to now sought to play down the problems at IMB. The official response to the Wissenschaftlicher Beirat's last report — which is said broadly to endorse Eigen's view and to call for far-reaching changes in the institute's structure and for Diekmann's resignation — was that "all is well".

Rigler says this was the last straw for him, and prompted his resignation as head of the scientific council. He says the most important issue now facing the IMB is the authority of the scientific director, who, he believes, should be able to control the institute's budget without the direct approval of the administrative director.

But officials in the Thüringen research ministry see things differently. "Making administration subject to scientific demands is a mad idea," says Klaus Bartholmé, deputy director of the ministry and head of the Kuratorium. "It could bring the institute to ruin."

In the light of the recent resignations, however, and with key scientists leaving what they consider to be a sinking ship, Bartholmé admits that the IMB is in serious crisis. He now says that the Kuratorium may be prepared to make changes to the institute's statutes along the lines that were suggested by Rigler and Eigen.

But many researchers fear that the reputation of the institute has been so damaged that changes may come too late, and the institute may be hard-pressed to fill the newly vacant posts with scientists of the quality it used to be able to attract.

Quirin Schiermeier



Eigen: resigned in protest at regime.

Sheep-dip chemicals claimed 'safe' if used by rule-book

[LONDON] Despite growing concern among sheep farmers, backed by some scientists, a British advisory panel last week issued a report stating that organophosphorus (OP) sheep dips are safe when used according to the manufacturers' instructions.

The report, by the veterinary products committee of the Ministry of Agriculture, calls for more basic research into OPs, ensuring that sheep-dip users are trained, and simplified labelling. The government has promised to act on the recommendations.

The report was prompted by growth in the number of sheep farmers reporting symptoms of apparent OP poisoning. Typically, a flu-like episode is followed by breathing problems, joint and muscle pain, fatigue, trembling, and mood swings.

The pesticides, used to control sheep scab by total immersion of the sheep in a solution, are known to cause neurotoxicological problems when in high concentration in humans. People in contact with the sheep-dip are advised to wear protective clothes.

The report's conclusion remains controversial. "The jury is still out over whether people can use OPs long-term without problems," says Alastair Hay, reader in chemical pathology at the University of

Leeds. Hay is unconvinced that poisoning symptoms are always explained by the absence of protective clothing.

Umbilical cord patent under challenge

[PARIS] The European Cord Blood Bank (Eurocord), a network of 14 research teams, has as expected (see *Nature* 382, 6; 1996) challenged a European patent granted to the US company Biocyte Corporation on the use of stored stem cells from umbilical cord blood. A separate challenge was simultaneously filed by a consortium of 30 environmental and other lobby groups led by Global 2000, an Austrian green group.

Both Eurocord and Global 2000 claim that the patent fails to meet the legal criteria of novelty and non-obviousness. Cord blood is a source of stem cells which can produce platelets and red and white blood cells, and promise to be simpler and more effective in bone-marrow transplants than using stem cells from donor marrow.

Support for labels on gene-altered food

[WASHINGTON] A leading biotechnology company has announced its support for the labelling of all genetically engineered crops and food products. Novartis, the Swiss chemical, agricultural and drug giant

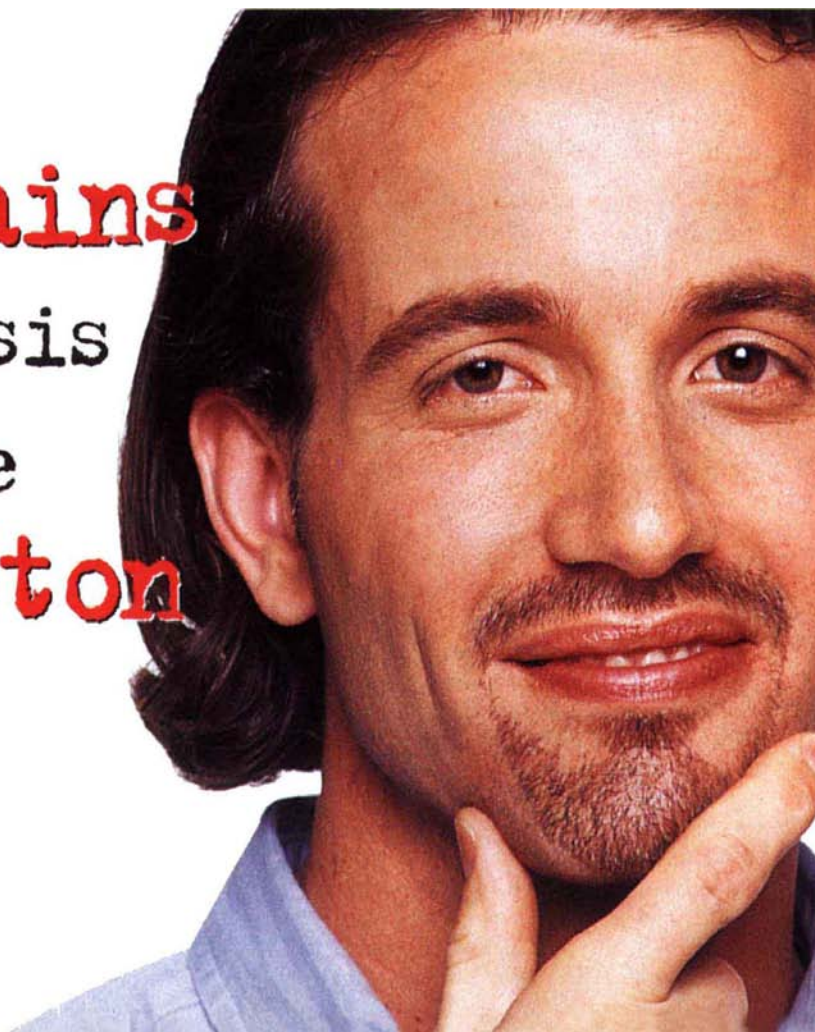
formed last year from the merger of Sandoz and Ciba-Geigy, outlined its position at a news conference in Boston, Massachusetts, on 24 February. Company officials acknowledge that there is no need for labelling from a scientific and safety standpoint, but say that if the industry believes in the consumers' right to choose, it "cannot reasonably argue against labels facilitating this choice".

Nobel laureate receives one-year sentence

[WASHINGTON] Daniel Carleton Gajdusek, the Nobel prizewinning virologist, pleaded guilty last week to a charge of sexually abusing a boy he brought from Micronesia to live with him in his home not far from the National Institutes of Health in Bethesda, Maryland. Gajdusek, who is 73, was until last week chief of the Laboratory for Central Nervous System Studies at the National Institute of Neurological Disorders and Stroke.

Gajdusek will serve nine to 12 months in Frederick County jail, followed by five years' probation, during which he may leave the United States. He was arrested last April. His plea agreement with state prosecutors, under which two counts of unnatural and perverted sexual practices were dropped, allowed him to avoid a potential 30-year prison sentence.

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Beyond the language barrier

Sir—I disagree with the contention of Paul R. Sanberg and his colleagues in recent correspondence¹ that “regional and local foreign institutions should continue Japan’s approach of providing important English-language journals”. It is a bad excuse indeed to claim that “important discoveries” are only “rediscovered” a few years later in another country simply because of a so-called “language barrier”. The continued ignorance of scientific publication often has more to do with the attitudes of scientists and of abstracting/indexing service providers.

For example, the Institute of Scientific Information (ISI) covers only a small fraction of archaeological periodicals published in Britain and Ireland for its *Arts and Humanities Citation Index*, yet the many journals that are not covered contain predominantly English-language papers, including important reports on archaeological excavations and surveys². If a ‘language barrier’ is not the problem, then what can it be?

Perhaps intellectual imperialism is at work here. Ireland has many local and society journals, yet only four Irish periodicals are covered by ISI for its *Arts and Humanities Citation Index*³. Other bibliographical and abstracting services such as the on-line *Celtic Studies Bibliography* of the Celtic Studies Association of North America⁴, the *International Medieval Bibliography*, the *British Archaeological Bibliography* and the Royal Historical Society’s *Annual Bibliography of British and Irish History* all include Irish archaeological periodicals not covered by ISI. However, none of these bibliographies is as comprehensive as it ought to be. This is due to funding cutbacks that make it difficult for academic libraries to subscribe to a wide range of foreign and indigenous periodicals, and this has had a knock-on effect to bibliographers who use these libraries for their compilations. In the recession of the 1990s, subscriptions to bibliographies have also fallen, as in the case of the *British Archaeological Bibliography*, giving rise to concern that (in this instance) archaeologists in Britain and Ireland will not be able to keep up to date in their profession^{5,6}.

Although increased funding for libraries and abstracting/indexing services should augment scholars’ knowledge of published work in other countries, the view that publication ought to be only in English is a bad one. At a time when science is increasingly being criticized for all kinds of reasons, the promulgation of such a view only lends support to its detractors. Not only could the charge of academic imperialism be laid against the principle of

a monoglot publication practice but it would also mean that fewer members of the wider public in non-English-speaking countries would be able to read the reports published by their own scientists. This would inevitably lead to a decline in the political clout of scientists within these countries, because their work might come to be seen as irrelevant to the concerns of their respective nations. Furthermore, adopting a single language for scientific communication would reverse the progress in democracy gained when Westerners moved away from the patrician use of Latin for all academic, legal and religious endeavour towards a more inclusive use of vernacular languages instead.

G. Fewer

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Sir—As Sanberg *et al.*¹ point out, there is no doubt that the presence in Japan of English-language journals in science shows that Japanese scientists have become international in their outlook. But this does not show the whole picture. In my opinion, there are two mundane reasons for the existence of English-language journals in Japan.

First, the medical journals published by many of the 80 or so medical schools in Japan provide an easy route for faculty members in these institutions to accumulate publications in English without proper—or even any—peer review. Papers in these obscure journals may be accepted on the whim of an over-burdened editor lacking the expertise needed to assess the quality of submitted manuscripts.

Second, these medical journals also serve as a cash-cow to the ‘English industry’ in Japan. The majority of Japanese scientists (medical doctors included) offer good money for their manuscripts to be rewritten and edited. A few years ago, I received an invoice for nearly US\$500 for “improving the English” of a manuscript submitted to a local journal published by a reputable medical school. I had previously had manuscripts accepted by journals in the United States and Britain (including *Nature*) without any request for changes to the English.

Sachi Sri Kantha

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Sir—I have been among the authors of about 30 research papers in international journals published in Europe and the

United States, and have thus had the opportunity of seeing reviews from many referees. The research was essentially experimental and was conducted within the framework of accepted ideas and experimental techniques. From my own experience I have regretfully to agree with Franklin D. Rumjanek⁷.

When a research paper by unknown authors from an unknown university in a developing country such as India is presented to an international journal, it may be natural for a referee working in a highly evolved research culture in a developed country to be sceptical about the quality of the work. Such a sceptical attitude was evident in some (but not all) of the reports I and my colleagues received. This taught us to aim for a higher standard than the average for papers published in such journals and to accept with stoic equanimity what may have been unfair rejection of a paper.

I should add that the prejudices of some scientists in developed countries against the work of their economically poor peers is insignificant compared to the prejudice based on religion, region, mother tongue and caste that I see in my own country.

N. Umakantha

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Sir—I work in the translation service of a German research centre, and from time to time authors show me papers that have been returned by English-language journals with comments to the effect that the language is “poor” or “needs revision”.

Some of the papers have already passed through our hands, and in my opinion the English is unobjectionable. Such comments are particularly maddening if no errors are indicated on the manuscript, just a blanket condemnation.

Like Rumjanek⁷, I put this down to prejudice against foreign, or rather non-anglophone, authors. We now resubmit the same paper, with minor cosmetic revision, adding that it has been revised by a member of staff from the language service. This policy has so far met with resounding success.

Janet Carter-Sigglow

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A call to action from the lab bench

Basic researchers in Britain and elsewhere must become more informed about science policy and more active as campaigners if their profession is to survive. Useful lessons can be learned from colleagues in the United States.

Michelle Peckham

It is remarkable that so many working scientists, at least in the United Kingdom, take so little interest in science policy, even though it affects them directly. When I recently presented a poster on the subject at a meeting of Royal Society university research fellows, only 20 per cent of those handed a questionnaire about science policy issues responded. A survey into potential difficulties in obtaining research funding conducted by the Royal Society generated a similar response.

It is true that many scientists are aware of the 1993 government white paper 'Realising Our Potential', an influential statement on science policy. But few have read either the paper itself or the Technology Foresight reports for their areas of research which arose from it.

One frequent complaint is that scientists need to concentrate on their research and teaching, and do not have enough time to follow broader science policy debates. A few do follow such debates, but most ignore them. Many others feel that, as individuals, they are unable to influence policy, and therefore choose not to object publicly to decisions they disagree with.

Why should scientists be aware of recent changes in science policy? The main reason is that grants for basic research are hard to obtain these days, not only because of cuts in government spending, but also because funds are being redirected from scientist-led, basic research towards strategic goals.

Industry's interests

Less money is available for responsive-mode basic research, which most academics use to obtain research funding because research councils are forced to redirect money into initiatives such as Technology Foresight, and the ROPA (Realise Our Potential Awards) scheme arising from the white paper. Ian Lang, the President of the Board of Trade, recently announced that, of the £1.26 billion (US\$2.02 billion) research budget for 1996–97, £778 million (equivalent to 73 per cent of the research councils' budget) will be invested in programmes related to Technology Foresight. This has apparently been welcomed by much

of industry, as it identifies long-term research priorities. But are academic researchers prepared to accept that their research agenda is dictated by industry?

The ROPA scheme rewards researchers who have already collaborated successfully with industry, as researchers are only eligible to apply if they have previously won £25,000 of industry funding. But not only does it use money that could have been used for peer reviewed research grants, it also results in funding research that might well not have been funded that way. ROPA proposals are not peer reviewed, but decided by a committee, many members of which will not be expert in the area of the research proposals.

On top of this, basic research in British universities is also threatened by cuts in the money that universities receive through the higher education funding councils. Each university now receives support for research based on its score in the national Research Assessment Exercise conducted every three to four years (the most recent was completed in January). It also receives money via the capital equipment budget, allocated by the Higher Education Funding Council for England (HEFCE) largely on student numbers.

In 1995, the government cut funds for capital expenditure by a staggering 35 per cent, wrongly assuming that this could be made up from private finance initiatives. Partly as a result of the threat by vice-chancellors to introduce fees for students, hitherto taught free, and industry's refusal to pay up, the government has acknowledged its mistake, not implemented further threatened cuts and has partly made up the 35 per cent deficit.

The picture is certainly gloomy. If scientists do not start taking notice of recent changes in the way that science is run, it will be hard to resist the threat to basic research. Scientists must not only inform themselves about science policy, but actively lobby parliament for the science policies they want. In the United States, the Federation of American Societies for Experimental Biology (FASEB) employs a lobbyist to represent its interests to Congress. The goal is to promote biological research to government and the public.

Members of FASEB's constituent bodies

are asked to lobby their congressional representatives by e-mail, and newsletters keep them up to date about relevant issues, and suggest appropriate action. The recent 6.7 per cent increase of the US science budget appears to have been partly the result of such concerted, direct action by scientists.

In the United Kingdom, the number of individuals who are concerned with influencing science policy is much smaller. Many professional societies, including the Royal Society of Chemistry, Institute of Physics, Institute of Biology and the Royal Society, actively lobby parliament. But, although many smaller societies are affiliated to these larger groups, science policy issues are rarely reported on in their newsletters. So most scientists are neither informed about such issues, nor encouraged by their professional societies to become involved in them.

The need for lobbying

One independent body openly critical of government science policy is Save British Science (SBS). It was set up just over 10 years ago by a small group of researchers in reaction to cuts in research funding. SBS informs its members about policy issues, asks them to voice their opinions to the media, and has written a comprehensive document setting out how science should be funded. But its membership remains low at just 4,000, and most of its lobbying is done by its central committee.

Yet it is vitally important that more scientists become actively involved in science-policy. Scientific societies could coordinate lobbying of MPs, ministers and policy coordinators by individual scientists. These societies could also start to inform members about science policy, by reports in newsletters, and e-mails. However, an organization similar to FASEB is unlikely to work so well in the United Kingdom, as politicians tend to dismiss much of the activities of lobby groups as 'special interest groups'. It is also up to scientists themselves to devote at least some of their limited free time to keeping up to date on policy, for example by reading reports in magazines, and consulting information posts on the Internet sites (see Table 1).

It is vital that more, and younger, academic scientists start to become involved in policy debates, and attempt to persuade politicians to defend their interests. At best, it would improve science funding in Britain; at worst, it could be the only way that basic research in the UK — and elsewhere — will survive. □

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Table 1 Web sites containing policy information

Office of Science and Technology (OST)	http://www.open.gov.uk/ost/science.htm http://www.open.gov.uk/osthome.htm
Parliamentary Office of Science and Technology	http://www.parliament.uk/post/home.htm
Higher Education Funding Council for England	http://www.hefce.ac.uk/
Research councils	http://www.nerc.ac.uk/joint_res_councils.html
Royal Society	http://www.royalsoc.ac.uk/rs/
Save British Science	http://dspace.dial.pipex.com/sbs/

The world's oldest spears

Robin Dennell

Humans of 400,000 years ago were sophisticated big-game hunters. Complete hunting spears discovered in a German coal-mine puncture the idea that these people hadn't the technology or foresight to hunt systematically.

Just occasionally, an archaeological discovery leaves one speechless. Reasons usually concern the degree of preservation, the unexpectedness of the find, and its wider implications. The discovery of complete, unambiguous throwing-spears 380,000 to 400,000 years old at Schöningen in Germany, described by Thieme on page 807 of this issue¹, meets all these criteria.

Schöningen is one of many enormous, unsightly, opencast brown-coal mines that dot the German landscape. This one covers 6 km² and is over 100 m deep. The good news for scientific investigators is that the exposures reveal a long sequence of Middle Pleistocene deposits, including a succession of river channels dated by their pollen and microfauna to a series of Middle Pleistocene interglacial periods. These appear to be well-dated: the second channel, which held the spears, belongs to a newly recognized warm phase, the Reinsdorf Interglacial, and is around 380,000 to 400,000 years old. As the earliest well-documented archaeological sites in northern Europe are about 500,000 years old², the Schöningen finds relate to the initial colonization of northern Europe.

The bad news about active opencast mines is the rate at which archaeological evidence is destroyed. The mining at Schöningen is dominated by a monstrous 50-m-high rotary shovel-excavator, whose cutting wheel is 11 m in diameter; this cuts through several tons of deposit a minute, around the clock and throughout the year. Miraculously, Thieme's small team was able to expose these remarkable spears in time.

Nearby peat deposits of a similar age were already known to contain remarkably well-preserved remains of large and small animals, fresh-looking stone tools, and even wooden implements. These included short, notched pieces of wood which may be the oldest-known handles for holding stone tools, and a longer piece, 0.8 to 1.0 m long and sharpened at each end. This item might be a 'throwing stick', possibly for stunning small prey, or a stabbing spear for killing wounded or cornered animals at close range.

Although remarkable, these finds pale into insignificance when set against the discovery of three complete spears, each around two metres long, associated with stone tools, thousands of horse bones (often with butch-

ery marks) and a possible hearth. Wooden finds like these would be sensational if only 3,000 years old; finds a hundred times older are almost unimaginable. Almost unimaginable, but not quite: a fairly complete spear from the last interglacial (115,000 to 125,000 years ago) was found in 1948, inside an elephant skeleton at Lehringen, Germany; and the tip of what might have been a spear was found at Clacton, England, in 1911, in deposits comparable in age to those at Schöningen.

The implications of the Schöningen spears are no less extraordinary than the degree of their preservation. First, these are unquestionably spears, and second, as such, they must have been used for hunting large mammals. Why are these simple inferences so significant to palaeolithic archaeologists? The reason is simply that hunting has become profoundly unfashionable in discussions of the Lower, and even Middle, Palaeolithic over the past twenty years. Until the 1960s, stone tools associated with large mammal remains were routinely explained as indicating the butchery of animals that had been hunted. Important examples were the initial interpretations of the very ancient 'living floors', 1.8 million years old, at Olduvai in Tanzania³, and the alleged elephant hunters at the much younger, Middle Pleistocene sites of Torralba and Ambrona, Spain⁴. Then came a long reappraisal of how these stone tools and

hominid and other mammal remains were found together. This was driven initially by Brain's re-examination of the australopithecine deposits at Swartkrans, South Africa⁵, by Binford's criticisms of the assumptions and methodologies used at Olduvai⁶ and, later, by patient analysis of cut- and gnawing-marks, surface weathering, and skeletal-part frequency at Koobi Fora and Olduvai, often under the inspiration of the late Glynn Isaac⁷.

By the early 1980s, few of the claims for big-game hunting in the Lower and even Middle Palaeolithic could be substantiated, as the evidence was too disturbed, fragmented and poorly preserved to show deliberate, purposeful hunting. Scavenging by both carnivores and hominids seemed a more reasonable inference, and some even suggested that big-game hunting did not occur until the appearance of fully modern humans in the Upper Pleistocene⁸, about 40,000 years ago. To fit this picture, the Clacton and Lehringen spears were downgraded to digging-sticks or, imaginatively, snow-probes for locating buried carcasses⁹.

But the Schöningen discoveries are unambiguously spears: to regard them as snow-probes or digging-sticks is like claiming that power drills are paperweights. There is other new evidence for the use of spears in lower palaeolithic Europe: one of the finds from the remarkably well-preserved 500,000-year-old site of Boxgrove, England, includes a rhinoceros scapula with a circular hole that may have been made by a thrown spear. The Schöningen spears now provide unambiguous evidence that large animals were killed in this manner by 400,000 years ago.

The spears have other exciting implications. First, the time and skill needed to make them: each is made from the trunk of a 30-year-old spruce tree; in each, the end with the tip comes from the base of the trunk, where the wood is hardest; and each has the same

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

An ancient two-metre spear rests among the bones of horses, perhaps its victims.

proportions, with the centre of gravity a third of the way from the sharp end, as in a modern javelin. These represent considerable investment of time and skill — in selecting an appropriate tree, in roughing out the design and in the final stages of shaping. In other words, these hominids were not living within a spontaneous 'five-minute culture', acting opportunistically in response to immediate situations. Rather, we see considerable depth of planning, sophistication of design, and patience in carving the wood, all of which have been attributed only to modern humans.

Secondly, the Schöningen spears might help explain the earliest hominid colonization of Europe. The earliest firm evidence of European hominids is the 780,000-year-old TD6 horizon at Atapuerca, Spain; the oldest sites in northern Europe include Boxgrove¹⁰, about 500,000 years old. It would have been difficult for hominids to survive at this time in northern Europe, as the climate was usually colder than today's; and winter survival would always have been hard without effective storage facilities, given the short daylight hours in which to find, kill and butcher large animals. The key to survival might have been an efficient hunting technology, reliant on throwing-spears, and supplemented by short stabbing-spears for dispatching wounded prey. What would be fascinating to learn is whether the earliest hominids in southern Europe and elsewhere had the same ability, or whether it originated in northern Europe. Although wooden spears are most unlikely to survive elsewhere, the impact marks of spears against bone (as at Boxgrove) might, and so the quest is not impossible.

Finally, these spears would have been heavy, and best used by large, powerfully built people. The extremely robust Boxgrove tibia appears to have belonged to such a person¹¹; and Neanderthals — who presumably also used such spears — were considerably more robust than their modern counterparts. 'You are what you throw' may be at least as apt for summarizing the European lower palaeolithic as the old adage, 'you are what you eat'. □

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Particle physics

New physics at last?

David Miller

Two experiments, both at the HERA positron–proton collider in Hamburg, have seen unexpected signals which appear to be signs of new fundamental physics. It is hard to see how the standard model of particle physics¹ could explain them. The results^{2,3} were presented for the first time at a special seminar in Hamburg on 19 February.

The experiments, called H1 and ZEUS, are run by international collaborations of about 400 physicists each. They differ in many technical details, but both of them are designed to capture and measure as many as possible of the particles that are produced in the accelerator when circulating positrons with 27.5 GeV of energy hit protons with 820 GeV going in the opposite direction. The machine has been running since 1994, but only now have enough events accumulated for the signals to be taken so seriously.

In each of the unexpected events, a positron bounces back with very high energy at a large angle to its initial direction (Fig. 1). A jet of hadrons (particles such as protons and mesons that interact by the strong nuclear force) appears on the opposite side of the detector, balancing the positron's sideways momentum. In many ways the events look like deep inelastic scattering (DIS), the process that 20 years ago gave us the first direct evidence for hard point-like quarks existing inside the proton. We can imagine the incoming positron hitting one of the quarks (or any other hard object inside a proton) and bouncing out at a large angle. And because naked quarks are never allowed to escape from an interaction point, the quark turns itself into a jet of hadrons of just the kind observed.

If the events are interpreted like this, the experimenters can measure three variables for each event: the amount of momentum transferred, called Q^2 ; the scattering angle; and the mass M of the positron–quark system. Expected distributions of the three variables have been calculated from the standard model of DIS. They fit the bulk of the data well in the range of parameters where the standard model has been tested very thoroughly. But at the very highest values of Q^2 and M , both experiments see too many events. ZEUS has seen five, where the expected number is about one. H1 has seen 12, where the expected number is 4.71.

Neither result would be very convincing on its own, but taken together they are very hard to explain as chance fluctuations of the expected signal. What can the effect be?

The H1 events have an extra tantalizing property: seven of them are clumped together

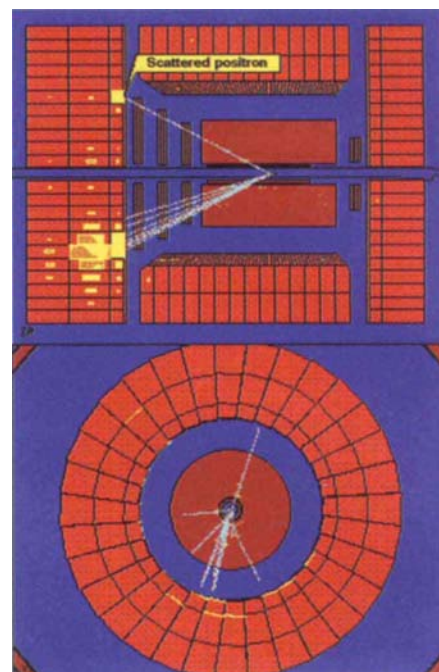


Figure 1 One of ZEUS's thunderbolts. In the side view (top), the scattered positron goes upwards and to the left from the collision point; the jet of hadrons goes downwards. The end view (bottom) shows their momentum balance. If a leptoquark was produced it decayed into these visible particles within a submicroscopic distance.

with values of M close to $200 \text{ GeV } c^{-2}$ (more than 200 times the mass of the proton), and the angular distribution of these events is much more even than in the rest of the sample, or in standard DIS events. If we take this clumping seriously then we might think that the events have been produced by the production and decay of a new short-lived particle. Instead of deep inelastic scattering this may be resonant scattering, in which the positron and the quark briefly merge into a single particle (not something predicted by the standard model) and then reappear when it decays.

That would explain the even angular distribution, because a particle with zero spin cannot carry directional information from its formation to its decay.

The resonant particle might be a 'leptoquark', with the properties (quantum numbers) of both the positron and the quark. Theorists like the idea of leptoquarks, but not at this mass. They are needed very early in the Big Bang as part of the only plausible theory⁴ that explains the excess of matter over antimatter in the visible Universe. But leptoquarks with a mass of only $200 \text{ GeV } c^{-2}$ would be embarrassingly light; they should already have caused all the protons in the Universe to decay.

Another resonance candidate comes from supersymmetry theories. But a very special kind of supersymmetry would be needed to make positrons and quarks stick together like this and then decay without any sign of missing energy. Most supersymmetry theories introduce a new, conserved quantum number, but these events would violate that conservation law.

Or maybe, since the ZEUS events seem to be more spread out, the clumping in H1 is just chance. In that case the underlying effect might not be the formation of a new particle but the action of a new force that causes a stronger interaction between the positron and the quark than anything in the standard model. This force might be carried by a massive leptoquark, or by a heavier version of the well-established neutral Z boson that carries part of the electroweak force. A neutral force carrier would explain why no extra events have been seen by H1 in the process where the outgoing charged positron is replaced by a neutral neutrino. A third possibility is that the HERA collisions are now probing so deeply that they have revealed a substructure to the quarks.

We may know which interpretation is right within a few years. Both experiments should double their number of events in 1997, and HERA will run until well into the next decade, with large improvements in intensity and the ability to scatter electrons instead of positrons from the proton beam which might allow different resonance particles to form.

Just now experimenters have the upper hand, and theorists are scrambling to find explanations — a change from the past 20 years, in which the standard model has withstood every challenge and many of its 18 parameters have been measured to better than one part in a thousand. A breakthrough was overdue: the values of the 18 parameters are as yet completely unexplained, along with many of the fundamental properties of the Universe at the earliest times.

A new level of understanding is required. It has to involve new particles and new forces at an energy scale beyond those reached by previous experiments. If the HERA results are indeed the first glimpse of these new particles or forces then the relevant energy scale is within our reach, at a few hundred GeV. This bodes very well for the success of the Large Hadron Collider at CERN, Geneva, which will reach an energy of 14 TeV by the year 2005. □

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Nuclear transplantation

An udder way of making lambs

Colin Stewart

A hoary old question that has interested developmental biologists for years has been the continuity of the genome during animal development. Put another way — do growth, differentiation and development of the embryo involve irreversible modifications to the genome in somatic cells? On page 810 of this issue, Wilmut and colleagues¹ extend their previous findings that viable lambs can be produced by transplanting nuclei from cell lines that have been established from very early sheep embryos (blastocysts) to enucleated eggs (Fig. 1). They now show that the same procedure can be used to obtain viable lambs from cell lines that have been established from older, more mature embryos and, remarkably, one viable lamb has been derived from a cell line that was established from the udder of a six-year-old ewe. The results are of profound significance. Not only do they confirm that the genome (with the exception of the immunoglobulin and T-cell-receptor genes) does not undergo irreversible modifications, but they will open up a range of new ways to think about the questions that are of current interest, as well as providing the impetus to develop novel approaches for the genetic manipulation of mammals.

Some 60 years ago, Spemann first raised the question of nuclear equivalency, or continuity of the genome during development. Since then, experiments (mainly on amphibians) have shown that nuclei from early embryos can be used to produce adult

clones. But nuclei taken from most tissues in tadpoles, or from adult tissues, have failed to produce viable adults, with the best-developing embryos rather ignominiously dying (croaking!) around the tadpole stage².

Attempts to extend cloning to mammals have also met with mixed results. Nuclei taken from mouse embryos older than the 8-cell stage, from totipotent embryonic stem cells (capable of giving rise to an entire, fertile organism) or from inner-cell-mass cells all fail to produce viable embryos^{3,4}. However, other species such as the cow and sheep have met with greater success, and adult sheep clones were the first to be produced using donor nuclei from early morula (8–16-cell) stage embryos⁵. Viable offspring have also been produced from nuclei taken from short-term cultures of pluripotent/totipotent cells, established from the embryonic disk of sheep and cow blastocysts^{6,7}.

So why can lambs be derived from later-stage embryonic tissues — equivalent to the frog's tadpole stage — or even from adult tissues? Clearly it is not because the cells are totipotent, although the udder cell that was used by Wilmut *et al.*¹ to provide the nucleus may possibly have retained some stem-cell characteristics. Rather, the key to success seems to have been in finding a method to make the donor nuclei more compatible with the cytoplasm of the recipient oocyte.

Nuclear transplantation studies in both amphibian and mammalian eggs indicated that a major problem in getting the trans-

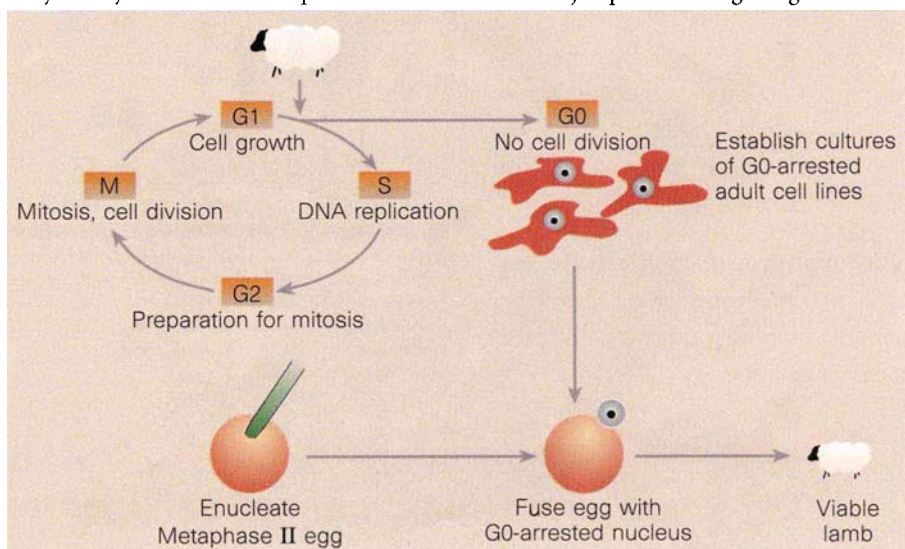


Figure 1 During the growth and differentiation of an embryo, do irreversible modifications occur to the genome in somatic cells? Wilmut *et al.*¹ have shown that they do not. By transplanting nuclei from a cell line created from the udder of a six-year-old ewe to an enucleated metaphase-II-arrested egg, they have created a viable lamb. Their success is largely due to the fact that nuclei were taken from cells in the G0 phase of the cell cycle — this prevents an additional round of DNA replication (as the G0 cells are essentially quiescent), and so reduces the incidence of chromosomal abnormalities.

planted oocytes to develop properly was cell-cycle incompatibility between the donor nucleus and recipient oocyte, with chromosomal abnormalities arising in the transplanted nuclei once development was initiated⁸. In mammals, most of the early embryonic nuclei that are used as donors are in either the S or G2 (non-diploid) phases of the cell cycle. This is not compatible with the diploid metaphase-II-arrested oocytes — the preferred recipient stage. When nuclei in S or G2 are introduced into metaphase-II-arrested oocytes, they tend to undergo additional DNA replication and premature chromosomal condensation, resulting in aneuploidy (whereby the nucleus is not diploid), and abnormal development⁸.

Wilmot *et al.* may have partially overcome this problem by transplanting nuclei from cells that have been arrested in the diploid G0 phase of the cell cycle by serum starvation. By arresting donor nuclei in G0, there should be better synchrony in the timing of DNA replication between the transplanted nucleus and the cytoplasm of the recipient oocyte once development is initiated, reducing the incidence of chromosomal abnormalities. It might also explain why it has been so difficult to clone mice: nuclei in the early preimplantation stages are mainly in S or G2, and it is very difficult (if not impossible) to arrest embryonic stem cells in G0 by serum starvation.

Other factors that may have contributed to the success of Wilmot *et al.* are increased access of chromatin from cells in G0 to oocyte 'remodelling factors' (presumably transcription factors and chromatin-binding proteins), and the fact that in sheep, transcription of the embryonic genome does not begin until the 8–16-cell stage, whereas in the mouse transcription occurs in the late 2-cell stage^{9,10}. This would, in theory, allow the sheep embryo at least two rounds of the cell cycle to reprogramme or remodel the transplanted adult nucleus to an embryonic state. But if these aspects are significant, it may not be possible to reproduce the results in other mammalian species, as the onset of embryonic transcription varies between species.

So what of the future? Nuclear transplantation from somatic cells could be used to produce clones of sheep that have been selected for particular traits; however, this may not be easy given the seasonality of sheep reproduction and the complexity of embryo transfer. Although the number of successful transplants is still extremely low, technical improvements will no doubt be made. Could the technique eventually be used to introduce modified genes into the sheep germ line? Current hurdles to this include the targeting frequency of genes in somatic cell lines versus embryonic stem-cell lines, the availability of isogenic genomic libraries, and the length of time that a normal karyotype can be maintained in cultured

sheep cell lines. Nuclear transplantation should also prove useful in studying the consequences of ageing on the function of the genome, and the impact of telomere shortening on senescence. Moreover, similar results could perhaps be obtained from the nuclei of neurons, or other cell types that have permanently withdrawn from the cell cycle. The success of Wilmot *et al.* in deriving a viable lamb from an adult cell will no doubt stimulate work to adapt or modify the technique to other mammalian species. Maybe, in the future, the collective noun for sheep will no longer be a flock — but a clone. □

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Phototransduction

Why lizards can't turn a blind eye

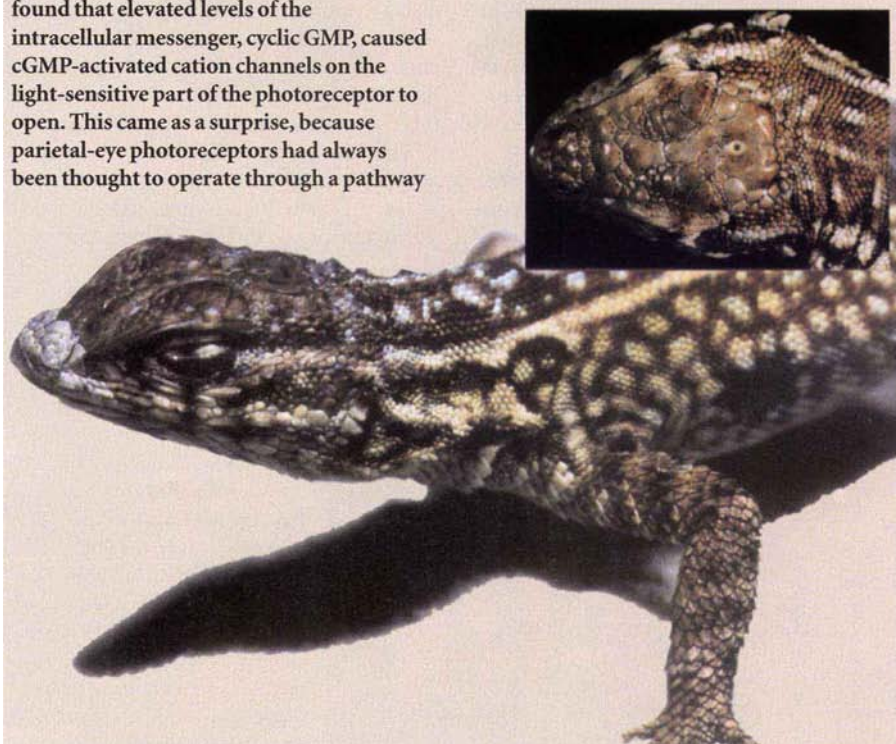
Most mothers would agree that an extra eye on the back of the head could be a useful device for keeping watch over their children. What they may not know is that many lizards and other lower vertebrates already have exactly this modification, in the form of a third, or parietal, eye. In the side-blotched lizard, *Uta stansburiana*, the parietal eye can be seen as a small dark spot (about 200 μm in diameter) underneath a translucent patch of skin at the midline — just behind the lateral eyes. For many years, photoreceptors in the parietal eye were thought to respond to light through a different intracellular signalling pathway from that used by the rods and cones in the lateral eyes. But in this issue (*Nature* **385**, 815–819; 1997), Finn *et al.* show that, in fact, the converse is true.

By recording the responses of parietal-eye photoreceptors to light, Finn *et al.* found that elevated levels of the intracellular messenger, cyclic GMP, caused cGMP-activated cation channels on the light-sensitive part of the photoreceptor to open. This came as a surprise, because parietal-eye photoreceptors had always been thought to operate through a pathway

that involved a different intracellular messenger, inositol trisphosphate. The new results bring the parietal-eye photoreceptors into line with vertebrate rods and cones, which also use a cGMP-mediated signalling cascade. But in this case, the levels of cGMP decrease in response to light, so the cGMP-activated cation channels close.

Finn *et al.* believe that their work may be of evolutionary significance, as all of the so-called 'ciliary' photoreceptors (a group that includes rods, cones, parietal-eye photoreceptors and hyperpolarizing photoreceptors in scallops) use a cGMP-mediated signalling pathway — so the chances are that if humans did have a third eye, it would respond to light by essentially the same mechanism as the other two.

Alison Mitchell



N. BARKER, M. McELWAIN & J. T. FINN

Many fingers make light work

Jonathon Mamin

As scanning-probe microscopes have evolved from the early 1980s, they have moved out of the research laboratory into the technological arena. This has largely been driven by the fact that the instruments have become so easy to use. Two reasons for this are the use of batch-fabricated micromachined cantilevers, and simplified schemes for measuring their deflection. But new applications are being envisaged, such as using probe tips for nanometre-scale lithography and data storage, that will require many devices operating simultaneously. The new level of complexity required will put a premium on approaches that simplify the overall design. Now Manalis and colleagues at Stanford University have made a new type of cantilever for scanning force microscopy that combines extremely simple optical detection with high sensitivity for the most demanding applications¹.

The scanning force microscope uses a sharp tip on the end of a sprung cantilever to detect and map out forces or topography over a surface. The ability to make an extremely soft cantilever and to measure its deflection at the ångström level allows one to work with very small forces. In the original design, a piggy-backed probe tip was placed a few ångströms above the back of the cantilever², and the deflection was measured by the electron current tunnelling from the tip to the cantilever.

But this type of displacement sensor, though highly sensitive, proved to be a bit painful to use in practice. For the less experimentally gifted, it became desirable to develop a simpler, more robust sensing scheme. Optical measurements are essentially non-perturbing and can be made remotely, so they offer a reliable alternative. Optical interferometry^{3,4} measures the difference in optical path length between a beam reflected off

the cantilever and a reference beam. It too is highly sensitive, but typically requires critical alignment of the optical components. Optical beam deflection⁵ is a simple though somewhat less sensitive alternative, requiring only a laser beam to be reflected off the backside of the cantilever into a split-cell photodiode. Because of its simplicity, it is the technique used in most commercial instruments. (Indeed, it is sobering that instruments that once were found in only a handful of the world's leading research institutions are now being sold as undergraduate teaching aids.)

The newest approach¹ combines the simplicity of optical beam deflection with the high sensitivity of interferometers. At the heart of the technique is a micromachined, movable diffraction grating, an idea previously proposed for high-resolution projection displays⁶. A laser beam hits the cantilever's interlocking fingers (Fig. 1). If the cantilever is not deflected, then it acts essentially as a mirror, and the light is mostly reflected. But if the outer cantilever is deflected relative to the inner support, then the differing heights of the fingers set up a grating, and the light is diffracted at well-defined angles.

This method simply requires a laser to be roughly aligned on the cantilever, and a photodiode to catch the diffracted light. In fact, the set-up is nearly identical to the beam-deflection technique used in commercial instruments, and is completely compatible with it. But because diffraction is inherently a form of interference, the technique has the higher sensitivity associated with interferometry. No apparatus need be close to the cantilever, so it could be used in awkward environments, such as liquids or vacuum. Finally, there is no need for a critical alignment of the diffracted laser spot onto

the photodetector as with beam deflection, because it is only the intensity of the diffracted light, rather than its position, that matters.

It has long been thought that scanning-probe techniques, with their ability to modify surfaces on the nanometre scale, might some day be applied to lithography or data storage. In fact, the group at Stanford have already made a 0.1- μm metal-oxide field-effect transistor using tip-based lithography with a single tip⁷. But one of the main problems is data throughput, since scanning-probe techniques are fairly slow. By operating an array of cantilevers in parallel, however, the throughput can be improved, and the group have now made arrays of cantilevers with integrated piezoresistive strain-gauges and piezoelectric actuators. They have also successfully patterned silicon using two tips operating simultaneously, with individual control of the loading force⁸.

In these devices the strain-gauge is integrated onto the cantilever itself, making it perhaps the simplest of sensors, with an electrical readout and no external components. That makes working with multiple cantilevers much easier, and it is an important step towards practical cantilever arrays. But the piezoresistive sensor is not yet as sensitive as optical methods such as interferometry. The interdigital cantilever¹ may be simple enough to use with arrays, and yet sensitive enough for more demanding applications.

The rapidly advancing field of micro-mechanics is making it possible to give scanning probes entirely new abilities. One can picture arrays of cantilevers with probes tailored for specific purposes. Already, new applications are emerging, such as femto-joule calorimetry⁹ and single-spin magnetic resonance¹⁰, that place the utmost demands on the cantilever and the detection scheme. While it remains to be seen what the technological impact of these advances will be, we can expect more innovations to add to the versatility and power of scanning-probe techniques.

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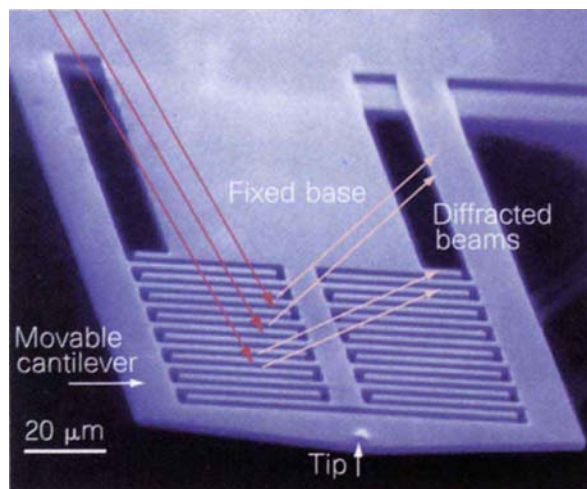


Figure 1 Micrograph of the interdigital cantilever¹. As in other atomic force microscopes, a tiny force on the tip of the cantilever makes it bend. But here, the grating formed by the interlocking fingers diffracts a laser beam, and the fraction of light in each diffracted spot depends on the deflection. Typically, the first two beams are about 1 mm apart at a distance of 20 mm — easily collected using a single split-cell photodiode. The method can detect displacement of order 0.01 Å, or less than 1% of an atomic diameter.

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Mutations make enzyme polymerize

M. F. Perutz

The formation of amyloid fibres from monomeric proteins moved to the centre of the scientific stage when it was found to be associated with Alzheimer's disease (AD) and transmissible encephalopathies such as BSE. We know little as yet about the native structures of the proteins which polymerize into fibres in AD patients, and we have the structure of only part of a mouse prion protein homologous to that in BSE fibres¹. Pepys and colleagues² have now determined the precise structures and properties of two mutant human lysozymes responsible for fatal amyloidoses (page 787 of this issue). The two structures do not explain why the mutant lysozymes form amyloid fibres, but they do suggest a parallel between the instabilities of the native protein structures introduced by the mutations in lysozyme, and by those in prion proteins which give rise to certain rare inherited amyloid diseases.

In 1853, the German pathologist Rudolf Virchow described eosinophilic waxy tissue deposits which he called amyloid (starch-like). The misnomer has stuck, even though all known amyloids are composed mainly of protein. Post-mortem brains of AD patients show extracellular neuritic plaques containing the 40–42-residue peptide fragment A β of a larger amyloid precursor protein, as well as intracellular double-helical filaments containing mainly the microtubule-associated protein tau. Brains of sheep affected by scrapie, and cattle affected by BSE, contain intracellular fibrils made of a protein called prion. Except in certain rare inherited diseases, the amyloid plaques and the prion

fibrils are made of the normal wild-type proteins. For reasons that we do not yet understand, these have become polymerized and transformed into β -sheets with their β -strands running normal to the fibre axis.

The two mutant lysozymes provide some clues. Both mutations affect internal residues. One of them replaces an aspartate — which forms a hydrogen bond with a tyrosine in a neighbouring β -strand — by a histidine. Such bonds can be very strong: rupture of an aspartate-tyrosine bond in an abnormal human haemoglobin raises the oxygen affinity by the equivalent of 3 kcal mol⁻¹. In lysozyme, its rupture opens a large gap between two β -strands. In the other mutant, an isoleucine attached to a β -bend is replaced by a threonine. The isoleucine side-chain is internal and forms a van der Waals contact with a neighbouring helix. The loss in free energy of stabilization of the native structure due to its replacement by threonine can be estimated from the difference in the solvation energies of the two amino acids on transfer from water to a non-polar solvent, which amounts to 2 kcal mol⁻¹ (ref. 3). The loss in free energy of stabilization of the native structure due to the loss of the tyrosine-aspartate bond is mainly enthalpic, and that due to the replacement of isoleucine by threonine is mainly entropic, but their disruptive effects on the native structure are similar. The losses in free energy may seem small, but they are large compared with the total difference in free energy between the folded and unfolded states of globular proteins, which is mostly less than 10 kcal mol⁻¹.

For comparison with the mutant lysozyme structures pictured on page 788, Fig. 1 shows the positions of the pathogenic amino-acid replacements in a fragment of a prion protein¹. Three of them lie between two helices where they may loosen the tertiary structure, like those of lysozyme.

The replacement of the aspartate by histidine lowers the temperature at which half the lysozyme molecules lose their catalytic activity by 14 °C, and the replacement of the isoleucine by threonine lowers it by 10 °C. The number of buried, exchangeable hydrogens replaced by deuterium after incubation with D₂O for 100 minutes at 37 °C is the same for the two mutants, and is nine times larger than in the wild type, confirming that the two mutations loosen the tertiary structure to about the same extent. The authors argue that these loose structures represent 'molten globules', but Oleg Ptitsyn, the inventor of this term⁴, defines this as a distinct metastable intermediate between the native and denatured structures, whereas the mutant lysozymes may merely

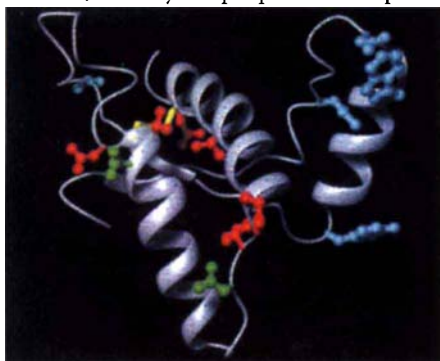
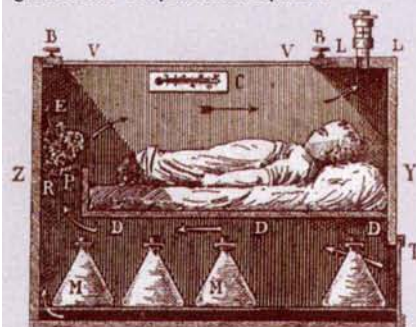


Figure 1 Booth *et al.*² have found that by replacing internal amino-acid residues within lysozyme, mutant proteins with an altered tertiary structure can be generated. Similar effects were observed by NMR studies of a fragment (residues 121–236) of a mouse prion protein¹, where certain amino-acid replacements lead to pathogenicity by 'loosening' the tertiary structure of the protein. The side chains of amino acids with mutations that have been found in inherited prion diseases are highlighted in red. (Reproduced from ref. 1.)



100 YEARS AGO

In a recent number of *L'illustration* an account is given of an incubator used for rearing delicate children. The apparatus designed by Dr. Tarnier, Professor of the Faculté de Paris, ... is constructed on the same principles as the incubator used for hatching the eggs of poultry. The apparatus, as first designed, consists of a large cubical box of thick wood, standing on a pedestal. This box is divided into two compartments, of which the lower contains a reservoir of hot water, and the upper the bed of the infant. A movable glass shutter forms the top of the apparatus, through which it is possible to observe the changes occurring inside, and take the readings of the thermometer placed near the infant. One side of the compartment is so hinged as to open like a door. The whole of the upper part is warmed by means of hot water underneath, the warm air rising through holes at each end of the bed, and escaping through orifices situated at the top. The temperature of the water is so regulated that the temperature of the apparatus never exceeds 30° to 37° Centigrade. The weaker the infant is, the greater the temperature required.



From *Nature* 25 February 1897.

50 YEARS AGO

Chromatography appears likely to play an important part in future work on protein analysis. The partition method, whereby amino-acids can be separated and identified on a cellulose strip, is sensitive to extremely small amounts of material. For example, 1 μ gm. of an amino-acid gives a clearly visible colour reaction with ninhydrin. The method may ultimately, therefore, prove valuable for the protein analysis of the chromosomes and their products. With suitable solvents and developers it should be possible to separate and determine the nucleic acids as well as amino-acids.

From *Nature* 1 March 1947.

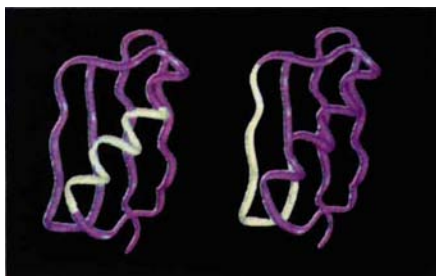


Figure 2 Ribbon diagram showing the conformation of the IgG-binding domain of protein G. On the left, the chameleon sequence is inserted in the position of the native α -helix, and on the right, the identical sequence is inserted in the position of the native β -sheet. (From ref. 5.)

fluctuate between the compact native and many different, partly open structures.

Minor and Kim⁵ performed a striking experiment which may be relevant to the prion problem. Using the immunoglobulin-binding domain of protein G, they engineered an eleven-residue peptide with a sequence that is a hybrid mixture between the sequence in a β -strand and that in an α -helix of the domain. They called this sequence a chameleon, because it folds as a β -strand when inserted at the position of the β -strand, and into an α -helix when inserted at the position of the α -helix of protein G (Fig. 2), showing that the conformation of the peptide adapted itself to the surrounding structure.

I wondered whether the sequences of the α -helices in lysozyme and in the prion protein show chameleon character. Except when they span membranes, α -helices usually have a mainly polar side facing the aqueous solvent and an interior side from which strongly polar residues are excluded. β -strands may be internal and all non-polar, or external with alternate polar and non-polar side-chains. I plotted the sequences along the α -helices in the prion protein fragment and in lysozyme, first on a helical net and then along a straight line with alternate side-chains pointing left and right. The five-turn helix 3 of the prion protein showed the expected distribution of polar and non-polar residues, but in the four- and three-turn helices, the distributions on the helical net seemed random. Nor did polar and non-polar side-chains alternate in any of the three segments as they should in β -strands facing the solvent. Perhaps the sequences in the four- and three-turn helices really are chameleons. Gasset *et al.*⁶ found that isolated α -helical peptides of hamster prion proteins form β -sheets, suggesting that their helical conformation requires stabilization by the intact tertiary structure of the prion protein.

Lysozyme contains five helices — three with three turns and two with only two turns. Short helices are intrinsically less stable than long ones, because a smaller fraction of their amides are hydrogen-bonded to each other. This applies also to the short helix in the prion protein. None of the helical sequences

in lysozyme shows either the outside-inside polarity of α -helices or the polar-non-polar alternation that is characteristic of β -strands. These helices may also be chameleons with only a marginal difference between the free energies of stabilization of the two conformations. Supposing one molecule unfolded to a mixture of β -strands and random coils, then another colliding with it might insert a β -strand between two β -strands of the first molecule, which makes its α -helices uncoil, and then a third molecule may insert a β -strand between two β -strands of the second, and so on. In this way, a process of crystallization would be initiated, driven by the lower free energy of the precipitate. But why do these mutant lysozymes and prion proteins assemble into ordered fibres instead of just forming amorphous precipitates like other proteins when mutations loosen their structure? This remains a mystery.

Extrasolar planets

One of our planets is missing

Gordon Walker

Just over a year ago, Michel Mayor and Didier Queloz¹ caused a sensation by announcing the detection of a four-day cycle in the radial velocity of the star 51 Pegasi, which they interpreted as a reflex motion induced by a 0.5-Jupiter-mass planet orbiting very close to the star. The variations were soon confirmed by others² and announcements of Jupiter-mass companions close, or very close, to seven other Sun-like stars quickly followed (they are all listed at <http://www.obspm.fr/planets>). It appeared that the Solar System, with giant planets in decade-long orbits, was exceptional, and that most giant planets lie close to their parent stars, in some cases even closer than Mercury is to the Sun. Theoreticians promptly offered plausible explanations³. But now, on page 795 of this issue⁴, David Gray has undermined the short-period planet interpretation.

From observations made at higher spectral resolution than in any of the discovery programmes, he has found a four-day variation in the *shape* of the spectral lines of 51 Pegasi, with enough amplitude to mimic the signature of reflex motion — so the planetary companion hypothesis is unnecessary. Gray's spectra also show changes, with the same period, in the ratio of two absorption-line depths that are sensitive to temperature in other stars. Although he can offer no single explanation for the line profile and depth variations, he emphasizes that the star itself must be varying periodically.

Where does this leave the other seven candidate giant planets? Before rushing into judgement, one must remember that detecting the signature of giant-planet perturbations is quite a challenge, and the line-

There is one crucial difference between the formation of amyloid fibres by the mutant lysozymes and prion proteins. Incubation of mutant with wild-type lysozyme produces fibres consisting solely of mutant lysozymes. On the other hand, infection of animals with wild-type fibrous prions of the same or a closely related species can make their own, normally stable, prion proteins become pathogenic. This is the crux of the prion problem for which a molecular model, amenable structural analysis and inhibition, has yet to be found. □

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profile changes in dispute are subtle and close to the limits of detection. It is impossible to take a direct image of a Jupiter-sized companion so close to its parent star, but the velocity perturbation is quite large (in the case of 51 Pegasi, $\pm 60 \text{ m s}^{-1}$). Changes in radial velocity are derived by measuring the shift in wavelength of absorption lines in the star's spectrum. Such lines for the Sun arise in the final 100 km of its atmosphere, and the Sun's rotation and convective motions broaden them to some $1,500 \text{ m s}^{-1}$ half-width. So the precision of 10 m s^{-1} required to detect planetary reflex motion needs a measurement accuracy of 1/150 of the line width. Several teams of astronomers have achieved this.

But — and it is a big but — the changes in velocity are only credible for the star as a whole if the spectral line shapes remain unchanged. Unfortunately, at some level, all stars are variable, which makes them poor wavelength standards. In the case of the Sun, large upwelling flows with typical velocities of 1 km s^{-1} can be modified by magnetic fields and spots, and differential rotation causes the spots to migrate, which will mimic net changes in velocity. The Sun also resonates like a huge bell over a broad range of frequencies around five minutes. Although the net effect of all of these variations is only a few metres per second, other stars are known to have more vigorous convective modulation, and some solar-type stars may be prone to longer-period, larger-amplitude pulsations.

Observations are limited by spectral resolving power. To adequately resolve a line of $1,500 \text{ m s}^{-1}$ half-width takes a resolution in wavelength of at least one part in 150,000, but the detections were made at typical resolu-

tions of 50,000. A resolution of 100,000 was enough for Gray to find an asymmetry in the 6,253-Å iron absorption line, similar to that caused by convection in other stars, with implied relative velocity of $\pm 45 \text{ m s}^{-1}$. That is close to the velocities measured by Mayor and Queloz, and it has the same 4.23-day period. Further, the ratio of the central depths of two lines that are normally sensitive to temperature fluctuations varies with the same period. The data extend over many years and are consistent over hundreds of velocity cycles. These results are convincing enough to give pause, but should we abandon the planetary interpretation altogether?

Gray's result raises the puzzle: what possible variability of 51 Pegasi could generate a perfect sine-wave in its radial velocities? A mechanism connected with rotation might do that naturally, but there is indirect evidence⁵ that the star's rotation period is about a month, far too long to cause the four-day variability. Perhaps any higher-order effects have been smoothed out by the low spectral resolution used in the Doppler detection or in the algorithm used to measure the line-shifts. Radial oscillations of the surface, on the other hand, would cause brightness fluctuations greater than those seen; and acoustic non-radial oscillations such as those on the Sun would have too short a period, and are unlikely to have remained coherent for hundreds of cycles.

Still, there has been ample evidence for some time that old solar-type stars — those that have evolved beyond the stable 'main sequence' — have a stable, low-amplitude velocity variability, with periods from days to years, which exactly mimics the signature of planetary reflex motion⁶. Gray points out that the absolute brightness of 51 Pegasi indicates that it is somewhat evolved, towards the sub-giant stage. Only by examining the spectral lines at sufficient spectral resolution can a 'Doppler map' be constructed of the star's surface using the star's rotation. Until such data are available, perhaps some time in the next year, we just can't be sure of the exact nature of any intrinsic variability.

So independent follow-up observations at even higher spectral resolution of 51 Pegasi and the other 3–14-day period candidates, ν Andromedae, 55 Cancri and τ Bootis, are essential. One startling outcome, if some of the short-period candidates have to be withdrawn, is that Jupiter-mass planets will become as rare as brown dwarfs, only two of which have actually been seen. □

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Conservation biology

Case studies of extinction

Paul H. Harvey and Robert M. May

The distributions of species are changing at unprecedented rates. Some are becoming extinct while others are expanding their ranges. To identify the general causes of these changes, as opposed to the particulars of individual cases, we need large data sets covering diverse areas.

Case¹ and Dobson *et al.*², writing in *Biological Conservation* and *Science* respectively, have now produced and analysed examples of such data sets. For island birds, for instance, they reveal that local extinctions are roughly matched by immigrations; that a high diversity of native species neither helps nor hinders the introduction of exotic species; and that exotic species find it much more difficult to invade native habitats than those modified by human activity. Of course, when the native species are endemic, the exotic species have come from elsewhere and global biodiversity is thereby reduced even if local avian biodiversity is maintained. We are moving towards an untextured world.

Case analyses three data sets for birds. The first is of 70 islands or archipelagos scattered around the world, plus two large continental land masses for which the number of native, established exotic and recently extinct native bird species can be estimated with reasonable accuracy. The number of native species is, as would be expected, well predicted by island area. But, although the number of extinct native species and estab-

lished introductions are well correlated with each other (accounting for about 44% of the variance in numbers among the islands), they are uncorrelated with either island area or the number of native species. This finding does not accord with Charles Elton's suggestion that more speciose communities are more resistant to invasion by exotics³.

As conservation biologists are well aware, exotics can become successfully established only if a viable population is introduced into an area. To what degree, then, is the number of established exotic species dependent on the total number of exotic species that could have established populations (either by natural invasion or by human importation)? In his second data set, Case identifies a subset of 22 locations for which these data are available. Across locations, there is a positive correlation between the number of species that have successfully established populations and those that have failed, which accounts for about 30% of the variance across locations in the number of successfully established exotic species. However, when the number of failures is controlled for statistically, the number of successes remains strongly correlated with the number of native species that have become extinct.

Are the native extinctions and successful introductions a consequence of native habitats being destroyed and subsequently replaced by new habitats to which native

Exhibition

Heard the one about the guinea pig?

This striking view of the inside of a guinea-pig inner ear is just one of the prizewinning images that are on display as part of the Biomedical Image awards at the Wellcome Centre for Medical Science in London. Although the spiral cochlea is only a few millimetres long, the photograph — taken by David Furness of the Department of Neuroscience and Communication at Keele University using scanning electron microscopy — reveals rows of sensory cells which respond to different frequencies of sound, running along the length of the structure. The winning images were chosen from new material acquired by the Wellcome Centre Medical Photographic Library, and the exhibition runs until 25 April 1997.

Allison Mitchell



species are less well adapted? To produce his third data set, Case recorded the populations of native and exotic species in natural and exotic habitats at ten locations. The proportion of native individuals or species in natural forest habitats was over 80%, but in modified grasslands and other non-natural habitats the proportion was reduced to around 50%. Case's literature surveys for New Zealand and Australia produced similar results to those stemming from his field work, although the proportions in the two countries differed somewhat because New Zealand has a far richer exotic bird fauna than does Australia.

Interestingly, using one line of reasoning, Case's finding that there is an approximate match between the number of native species lost and exotic species gained suggests that the new habitats have impoverished species diversity. We know that the number of species roughly increases with area raised to the 0.25 power. Other things being equal, this means that if part of a native habitat on an island is destroyed and replaced by new habitat which is invaded only by exotics, the total number of species on the island should increase.

Suppose, for instance, that the loss of 50% of native habitat on an island is matched by a gain in new habitat that can be exploited only by exotics, then in a simplistic limit we might expect the number of native species to diminish by 16% but the total number of species on the island as a whole to increase by 68% as exotics become established in the modified region. The fact that the total number of species remains approximately constant indicates that other factors are at work: we know that neither natives nor exotics are wholly confined to particular habitats; it is likely that the new habitats are not able to support as many species as the old habitats; and frequently there has not been enough time for sufficient exotics to establish themselves.

Preventing extirpation of species in the first place is, of course, to be preferred over reintroducing them. After all, in the second instance habitats must be re-created, often with reintroductions of species from a variety of taxonomic groups. So that limited resources can be optimally used, increasing effort in recent years has gone into identifying endangered species that are most under threat of extinction⁴.

This is the background to the paper by Dobson *et al.*². These authors use the endangered species lists for both animal and plant taxa occurring in the various counties of the United States of America (and therefore including Hawaii) to determine whether hotspots for endangered species coincide across taxa, and to help identify those areas most in need of conservation resources. Using a complementarity algorithm, they first identify the county with most endan-

gered species, then the county with most endangered species that are not included in the first county, and so on. Most endangered species occur in Hawaii, southern California, the southeastern coastal states and southern Appalachia. Their complementarity algorithm also selects counties in those same regions. Overall, between 1% and 5% of total land area delimits those counties which are required to sample more than 50% of endangered species for particular taxa.

In Britain, which is smaller and less speciose than the United States, Prendergast *et al.*⁵ found that the locations of hotspots for one taxon are not good predictors of locations of hotspots for other taxa. Dobson *et al.* come to the same, unfortunate conclusion. For example, they applied the complementarity algorithm to birds to identify the minimum number of counties required to locate all endangered species. That area contained only 30 to 40% of endangered herptile (amphibian and reptile), mammal and plant species, just over 10% of arthropod and fish species, and about 2% of mollusc species.

Would it, then, be possible to find a taxon that is a much better predictor than birds of the presence of endangered species? At first sight the answer appears to be plants. By identifying the minimum area required to conserve all plant species, we would also be conserving 94% of birds, and between 39% and 76% of species in other taxa. The downside to this is that the area containing all the plants is almost 10% of the area of the United States, whereas that for birds is less than 2%.

Dobson *et al.* conclude that birds are, in fact, probably the best indicator species for other taxa when constraints of land area have to be taken into account. As with other hotspot studies, it is possible to identify critical areas for some combinations of taxa, but others tend to be excluded. As an unsurprising example, locations containing threatened species in water bodies are not necessarily found in the same areas as those containing threatened terrestrial species.

Nevertheless, the general patterns revealed by Case, Dobson *et al.* and others are beginning to give us a better understanding of the general factors causing both the loss of native populations and the successful invasion by exotic species. Only through that understanding can we hope to slow down, halt or even reverse those processes. □

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Daedalus

The sap also bubbles

How does water get to the top of a tree? Botanists claim that it is sucked up by its evaporation at the top. For trees taller than ten metres, however, the liquid xylem must be sucked so hard that it experiences negative pressure, that is, tension.

This is not quite as absurd as it seems. In clean conditions, a column of liquid can withstand many atmospheres of tension before it breaks, cavitating into bubbles of vapour. This could be checked by measuring the xylem tension in a tree. Daedalus once proposed to cool down a section of tree, to measure the freezing point of its xylem. This is lowered by pressure, and must be raised by tension. The moment of freezing could be detected by ultrasound or X-ray diffraction. Sadly, ultrasound might itself cause cavitation, relaxing the tension to be measured, while X-rays could create bubbles in the xylem, bubble-chamber fashion.

But Daedalus now argues that this must happen all the time anyway. Cosmic rays and wind vibration must constantly cavitate the xylem in trees. To live at all, they must have some mechanism for compressing and collapsing the bubbles in their xylem as fast as they form. Perhaps the xylem ducts have pressure-sensitive valves at each end, which detect cavitation. When they do, the valve at the top opens, the one at the bottom closes, and the xylem above the bubble slowly descends to collapse it. Pumping then resumes, and the metabolism of the tree is soon restored.

So Daedalus now sees his erstwhile experiment as a powerful way to control the growth of a tree. In parks, gardens, town squares and landscaped grounds, trees need constant attention. Foresters and tree surgeons must always be cutting them back, maintaining their elegance while keeping their branches from overshadowing lawns, or their roots from damaging foundations.

The DREADCO xylem cavitator will change all this. Attached to a tree, it will launch a succession of ultrasonic pulses, or a well-judged trickle of high-energy particles, into its trunk. It will challenge but not overwhelm the tree's cavitation-repair system. With proper calibration, it will not kill the tree, but will exactly arrest its growth. Smaller versions could be attached to specific branches, sabotaging their growth while allowing others to extend without opposition. The tree could thus be sculpted into any desired shape. With no sawing or mutilation, ornamental trees will be formed to perfect shape and condition, making silviculture an even finer art.

David Jones

Clyde Tombaugh (1906–97)

Astronomer who discovered the Solar System's ninth planet

Clyde William Tombaugh died on 17 January at his home in New Mexico; he was 90. His major research achievement, discovering the planet Pluto, precipitated one of the most daunting puzzles of twentieth-century planetary science — a puzzle that Tombaugh fortunately lived to see largely resolved in the final years of his long life.

Tombaugh was born in 1906 in Streator, Illinois, to a family of wheat farmers. At that time, both Percival Lowell and William Pickering were undertaking the first searches for a planet suspected to exist beyond Neptune on the basis of perceived irregularities in the orbits of Uranus and Neptune. Five such attempts, carried out up to 1916 when Lowell died, were all unsuccessful.

Tombaugh developed an intense interest in astronomy during his adolescence, at first borrowing and later building several small telescopes. By the late 1920s he was regularly corresponding with astronomers at the Lowell Observatory in Flagstaff, Arizona — eventually earning himself a job at Lowell as an observing assistant for the renewed search for the suspected planet (dubbed 'X') to begin in 1929. When Tombaugh arrived in Flagstaff in January 1929, he was just 22 years old, had no formal training in astronomy, and only a high-school education.

The search technique for Planet X would use a new, wide-field, 13-inch telescope and camera, and was based on the fact that planets move against the background stars. Photographs of star fields would be made and compared using a special device called a blink comparator, which allowed the operator to distinguish between stationary and moving objects. The necessary equipment was ready in April. Within days of starting, the observatory's director, V. M. Slipher, left Tombaugh to his own devices to carry out the search.

Tombaugh adopted a schedule of sky photography during the period each month around the new Moon, when the sky was darkest; data analysis was undertaken when the Moon was more or less full. He contributed significantly to the search protocol by always obtaining a third sky plate of every field to help confirm or reject potential finds. He also realized that by searching the opposition point (the patch of sky opposite to the Sun), the amount of

motion a moving object showed would be directly related to its distance. In this way, he was able to distinguish comparatively nearby (hence more rapidly moving) foreground objects, such as asteroids, from the hoped-for, distant, trans-neptunian planet.

The task was not easy. There are over 15 million stars in the sky brighter than Pluto. Each of the 170-plus plates Tombaugh obtained in his initial search contained between 50,000 and 900,000 stellar images, plus the tracks of many asteroids. Barely ten months into the search, however, Tombaugh found his prey. On the afternoon of 18 February 1930, while comparing plates of the constellation Gemini, his eye detected a faint pinpoint of light that jumped 3.5 millimetres — in accord with a planet moving two billion kilometres beyond Neptune. "That's it", he later recalled saying to himself, and confided that he briefly savoured the fact that he alone among humanity knew that a ninth planet indeed existed. He then proceeded to Slipher's office, where he calmly announced, "I have found your Planet X".

Following three more weeks of observations and checking by Lowell staff, the discovery was announced on 13 March 1930 — the 75th anniversary of Lowell's birth, and the 149th anniversary of Uranus' discovery by Herschel. Shortly, 'X' was named Pluto, after the Greek god of the underworld, and in honour of Percival Lowell (P. L.).

After the discovery, Tombaugh was awarded a scholarship and took astronomy degrees at the University of Kansas. He married in 1934, and continued to search for planets from Lowell during the 1930s and early 1940s. He found none, but his sky survey did reveal numerous new asteroids, a comet, a nova, a globular cluster, five open clusters, and the Andromeda–Perseus supercluster. During the Second World War, he taught navigation for the US Navy, and

afterwards became involved in early rocketry at White Sands Proving Grounds. In 1955, Tombaugh joined the faculty of New Mexico State University, where his research included a search for small satellites of Earth, studies of Mars that correctly concluded in 1950 that Mars would have impact craters, and studies of galaxy clustering. He retired in 1973, and remained an inveterate punster and a man of great energy. When the Smithsonian Institution recently asked him to contribute his 9-inch telescope to its collections, he responded, "They can't have it — I am still using it".

Ironically, by the 1970s, it was established that Pluto is too small to be the perturber of Uranus and Neptune that first set off the search. It later also emerged that those perturbations stemmed from inaccurate measurements, and were themselves fictitious. The search for X had been based on false premises.

Despite this, as more was learned about Pluto, its nature and context within the Solar System became increasingly puzzling. Its highly inclined (16°) and elliptical (25%) orbit is unlike that of any other planet. Although it crosses inside the orbit of Neptune for a brief portion of each 248-year orbit, the planet is protected from ejection by massive Neptune owing to a unique orbital geometry and a set of overlapping dynamical resonances with Neptune. The planet has a tiny mass (only a tenth that of the Moon), a diameter of just over 2,300 km, and a rotation axis inclined 120° to its orbit plane.

Pluto is also distinguished by a complex *mélange* of exotic surface ices and by its satellite, Charon — with a diameter half as large as Pluto. The pair are the only known example of a true double planet.

For decades, planetary astronomers were puzzled by the odd fact of a tiny, binary world lying on a strange orbit beyond the giant planets. Since 1992, however, a plethora of other, far fainter worlds have been discovered in the region where Pluto orbits. This discovery of the Edgeworth–Kuiper Belt provides a *double entendre* to a remark that Gerard Kuiper once made to Tombaugh, that "What you didn't find is more important than Pluto".

It is fitting that Tombaugh lived to see his discovery placed in its true context as the bright tip of a huge underlying population of remnant icebergs, planetesimals and small planets lying beyond the giant planets — in many respects reminiscent of the planetesimal disks discovered in recent years around other stars.

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A new look at old sharks

Here we describe what we believe is the most complete three-dimensional fossil shark yet discovered. The fossil (classified in the genus *Tribodus*, a member of the extinct family Hybodontidae¹) is from the Romualdo member of the Santana Formation in northeastern Brazil (from the Albian stage of the Cretaceous period; roughly 110 million years old)². It provides an unparalleled opportunity to examine the braincase, jaws and hyoid arch of an ancient shark.

Tribodus shares many anatomical features with other hybodont sharks³, including the presence of large, barbed denticles (cephalic spines; see Fig. 1) on each side of the head in males. These spines are an evolutionarily advanced secondary sexual characteristic of hybodont sharks. Until now it was not clear how they were attached to the head. In *Tribodus* the convex base of each spine rests diagonally across a slightly concave fibrocartilaginous platform on either side of the braincase otic region. A comparison with other male hybodonts with preserved spines indicates that these were probably similarly attached.

We also note several differences between *Tribodus* and other hybodont sharks. For example, *Tribodus* lacks a suborbital shelf. The region under the eye is almost completely surrounded by the preorbital and postorbital processes. It also lacks a median keel in the ethmoid (snout) region. A prominent ventral process below the orbit probably anchored a ligamentous attachment for the palatoquadrates, but there are no direct articulations between the jaws and braincase. Another curious feature is the presence of paired, calcified projections from the floor of the otic region (not shown in Fig. 1). No other living or extinct shark has comparable structures, and their function is still to be determined. The large orbits (of similar size to those in modern thresher sharks) suggest good visual acuity or adaptation to low light levels.

The jaws do not extend beneath the snout, and are entirely supported by the hyoid arch (hyostylic jaw suspension). The ray-like dentition of closely spaced, polygonal teeth forms a flat pavement, and the median skeletal element of the hyoid arch (basihyal) forms a wide transverse bar. These are all advanced evolutionary features that are also found in many modern sharks and rays, but pending a more comprehensive cladistic analysis (J. G. M. & M. R. d. C., manuscript in preparation), they are regarded as convergent evolutionary trends rather than features uniting *Tribodus* with any particular recent group.

Olfaction was probably important in prey detection and feeding. The entire

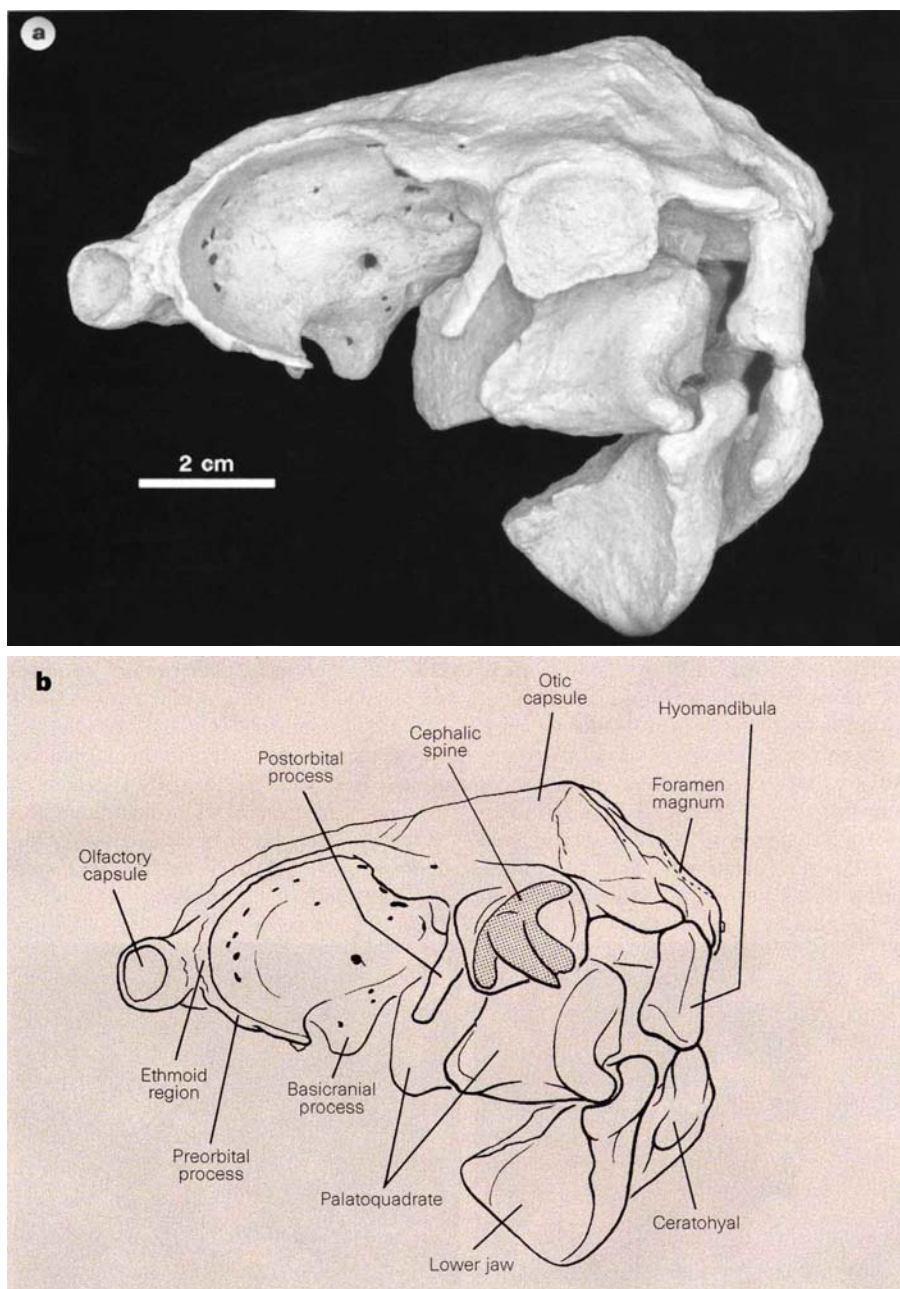


Figure 1 a, Mounted cast of the braincase, jaws and hyoid arch in *Tribodus* seen in left lateral view. (Specimen AMNH 13958, Axelrod collection; prepared by R. Evander, American Museum of Natural History.) Note the basicranial process below the large orbits, the short hyostylic jaws, and the narrow postorbital process that projects downwards in front the palatoquadrate, which has a deep fossa for the jaw adductor muscles. b, Annotated line drawing (by L. Meeker) showing the true position of the cephalic spine.

braincase is inclined downwards relative to the long axis of the body, and when swimming these sharks could have brought their olfactory capsules close to the substrate to detect benthic organisms before ingestion. The presence of fossil shrimps and coarse sand grains in the body cavity of another specimen of *Tribodus* provides compelling additional evidence that these sharks were benthic feeders.

Hybodont sharks first appeared in the Late Palaeozoic era and reached peak abundance during the Jurassic period. They were gradually replaced by modern elasmobranch families and apparently became extinct during the Late Cretaceous period¹. *Tribodus* offers a tantalizing glimpse of previously unsuspected morphological diversity among hybodont sharks from the 'age of dinosaurs', and indicates that the range of

feeding and related behavioural strategies among hybodonts might have been as diverse as among living sharks and rays.

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A pivotal Archaea group

Barns *et al.* have identified two novel Archaea sequence types in an analysis of organisms from a hot spring in Yellowstone National Park¹. These types are different from all known archaeal sequences, and seem to represent a pivotal group, Korarchaeota, in the phylogenetic tree². The ability to culture and to identify these organisms is critical for understanding the evolution of the Archaea and Eukarya.

To cultivate the organisms in the laboratory, we took anaerobic water and sediment samples (pH 5.5; 70–90 °C) from Obsidian Pool, a hot spring in the mud volcano area of Yellowstone National Park, Wyoming¹. We set up a continuous culture at 85 °C in the laboratory (Fig. 1). After about 1 week,

we found roughly 2×10^8 cells per ml of very complex community with heterogeneous morphologies (cocci, rods and filaments). We determined that the appearance of the community remained stable for more than a year, using phase-contrast microscopy. Whole-cell hybridization with fluorescently labelled oligonucleotide probes targeted to Archaea- and Bacteria-specific regions within the 16S ribosomal RNA^{3–5} reveals that most of the organisms (more than 90%) belong to the Archaea.

Korarchaeota have, so far, been detected only by analyses of 16S rDNA sequences obtained directly from the environment^{1,2}. To detect members of this new archaeal kingdom in the mixed culture, we isolated DNA twice monthly for more than a year, and used it in polymerase chain reactions with primers highly specific for the 'korarchaeal' 16S rRNA gene (Fig. 1). All isolated DNA produced sequences identical to the korarchaeal sequence type pJP27 (GenBank accession no. L25852).

We determined the morphology of the Korarchaeota by whole-cell hybridizations^{3,6,7} using different fluorescently labelled oligonucleotide probes complementary to three regions of the korarchaeal 16S rRNA sequence (Fig. 1). We checked the sequence specificity of the probes by comparison with all 16S rRNA sequences available in databases, including the sequences obtained by *in situ* analyses of the Yellowstone hot spring^{1,2}. We also evaluated the specificity of the probes by hybridizations of pure cultures from known hyper-

thermophilic Archaea and Bacteria. Only those cells reacting to two differently labelled korarchaeal probes in a single hybridization experiment were identified as Korarchaeota.

As a positive control for the other organisms present in the culture, we used a universal probe in hybridization experiments. Very few cells (about 10^4 – 10^5 cells per ml) gave a positive hybridization signal with the korarchaeal probes. The organism corresponding to the sequence type pJP27 is rod-shaped, variable in length (between 5 and 10 µm) and most of the cells are slightly curved (Fig. 1). The diameter of the cells is about 0.5 µm. We detected dividing, actively growing cells. The number of these cells within the 85 °C mixed culture was stable for more than a year, indicating that this member of the Korarchaeota is a hyperthermophile.

To characterize the Korarchaeota, it is crucial to grow these organisms in the laboratory. Here we have shown that they can be cultivated as a stable population in a mixed laboratory culture. Isolation of the Korarchaeota into a pure culture for study of their biochemical and physiological properties in detail should improve our understanding of phylogenetic relationships during the early stages in the evolution of life.

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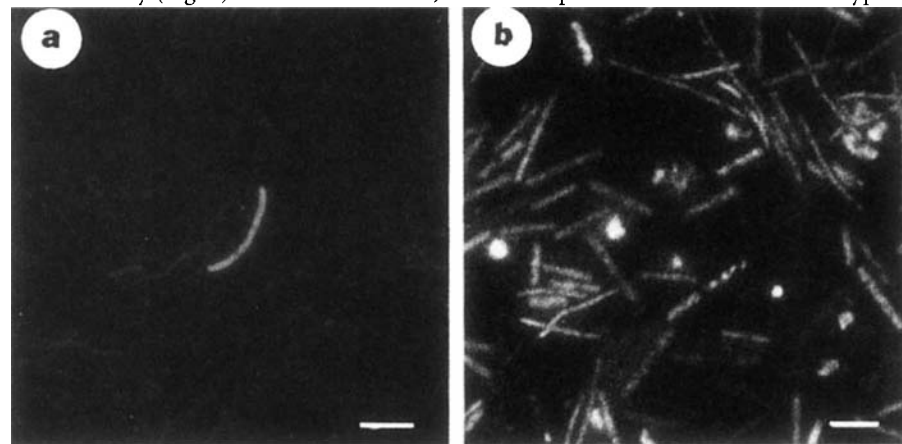


Figure 1 Micrographs of the mixed culture at 85 °C, taken on a Nikon Microphot EPI-FL microscope. Scale bars, 5 µm. a, Whole-cell hybridization with the fluorescently labelled probe 1135R (5'-GTTTG-CCCGGCCAGCCGTAA-3') specific for Korarchaeota. Similar results were obtained with the probes 604R (5'-TGTCTTCAGGCGGATTTAAAC-3') and 546R (5'-AGTATGCGTGGAACCCCTC-3') and combinations of these probes labelled with different fluorescence dyes. b, Diaminophenylindole-stained cells, showing the different morphotypes present in the culture. The primers used in the polymerase chain reaction to amplify 'korarchaeal' 16S rDNA specifically were 5'-GAGGCCCGAG-GRTGGGACCG-3' (236F) and 5'-GTTTGCCCGGCCAGCCGTAA-3' (1135R). For cultivation, we placed ~400 ml of anaerobic water and sediment sample and ~400 ml anaerobic original spring water, supplemented with 0.001% yeast extract, 0.005% peptone and 0.005% Na₂S₂O₃ into a two-walled glass vessel (chemostat). We heated the sample to 85 °C by pumping heated glycerol between the two walls of the chemostat. The chemostat had a total volume of ~800 ml and was 'aerated' with N₂ and CO₂ (80/20 v/v, 20 ml min⁻¹). Later we substituted the original spring water for an anaerobic, low-salt medium (dilution rate, 20 ml h⁻¹) containing the same nutrients⁸.

Fullerene 'crop circles'

While examining laser-grown single-wall carbon nanotube (SWNT) material^{1,2} by scanning force and transmission electron microscopy, we regularly observed circular formations of SWNT ropes (Fig. 1). These ropes consist of between 10 and 100 individual nanotubes aligned over their entire length, packed in a two-dimensional crystalline array. Sceptical that these objects might be perfectly seamless toroidal nanotubes, we named them 'crop circles', but we are now convinced that many, perhaps most, of the individual tubes in these circular ropes are indeed perfect tori.

We estimate that between 0.01 and 1%

of ropes are of this circular type. Diameters typically range between 300 and 500 nm, and rope widths, 5–15 nm, are similar to those of ordinary ropes. Transmission electron micrographs of ‘crop circles’ clearly show fringes corresponding to individual tubes (Fig. 2). The scanning force microscopy (SFM) image in Fig. 1 shows a particularly thin circle, with uniform height and width (apart from local variations arising from small quantities of amorphous carbon). The circle shown in Fig. 1 has a height of only 1.0–1.2 nm, and is almost certainly a single continuous toroidal nanotube, with no beginning or end. There is no evidence in the SFM image of a step that would result from the start or end of a coil.

The fact that typical cross-sections of circular ropes are in the same range as SWNT ropes further compels us to conclude that many, perhaps most, constituents of the circles are perfect tori. If the circles consisted only of coils, then they should be much thicker, because roughly 100 turns would be required to produce a total length similar to that observed in rope fibres¹. The observed rope width of the circles is, however, consistent with the formation of an initial torus, which may serve as a template for subsequent circular growth from tube nuclei deposited from the vapour. Although we cannot rule out the existence of coiled tubes in these circles, it is likely that there are at least some continuous tubes with no ends.

To explain the formation of these toroidal fullerenes, we propose a Kekuléan image of a growing nanotube eating its own tail. The first step, in which a single tube bends around to touch near its ends, is not yet well understood (although one plausible mechanism has been proposed³), but subsequent steps in the formation of a torus can be explained more readily. After the ends of the tube have touched, they align to maximize van der Waals interactions, but slide over one another to alleviate bending strain. Usually they will slip out of contact, but occasionally the closed hemi-fullerene end will stick to a metal particle (added as a catalyst when making the nanotubes) that becomes attached to the growing end of the tubes. At the 1,500 K growth temperature, the two ends become welded together by the metal plug. They may be able to knit together seamlessly, annealing to the perfect toroidal tube structure, at which time the now useless metal particle is shunted either to the exterior or interior. With no edges, there is no further growth of the torus.

This picture of facile connection of ends may explain how ‘regular’ rope growth terminates, and why rope ends are almost never observed in high-yield SWNT material. During the growth phase, after nucleation, a rope end colliding with the side of another rope might align to maximize their van der

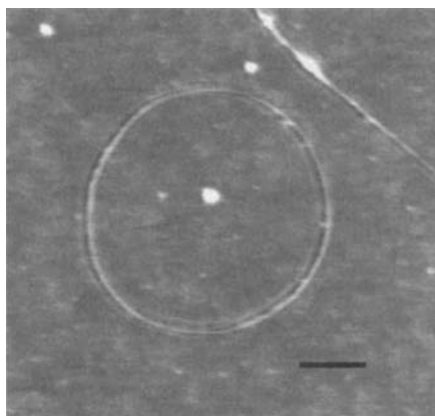


Figure 1 Scanning force micrograph of a ‘crop circle’, imaged with a pyramidal SFM tip (the effect of a double tip can be seen at the top and bottom). The circle has an apparent height of 1.0–1.2 nm and width of 4–8 nm. This falls to the lower end of the distribution of tube diameters in this sample, but pyramidal tips produce artificially small circle heights², a problem from which nanotube tips suffer little. Thus, the actual height of this tube is probably closer to 1.5 nm, a more typical SWNT diameter. SWNTs were prepared as described previously¹. Transmission electron microscope and SFM samples were prepared from a drop of SWNT material sonicated in acetone. Scale bar, 100 nm.

Waals interaction. As it continues to grow on its metal ‘chaperone’, the rope end may eventually encounter a third rope growing in the opposite direction, and by the mechanism described above, fuse into continuous tubes.

Single-wall carbon nanotubes are very promising new materials with unusual properties. The metallic subclass of ‘armchair’ SWNTs, such as the archetypal (10,10) tube¹, constitute genuine quantum wires, owing to their nanometre-dimension diameters^{1,4,5}, thus electron transport in these materials should prove interesting both for the fundamental understanding of quantum wires, and for the development of new devices.

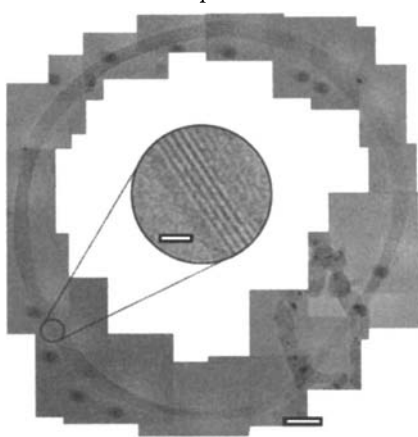


Figure 2 Transmission electron micrograph of a ‘crop circle’, showing fringes with spacings typical of SWNTs in normal ropes. Scale bars, 15 nm; inset, 5 nm.

Transport behaviours (for example the Aharonov–Bohm effect) in these toroidal fullerenes should now be studied.

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Plants combat infection by gene silencing

Plants with resistance genes can combat viruses by eliciting an incompatible interaction at the site of infection. Resistance can also occur in plants containing transgenes that share homology with an infecting virus^{1,2}, by the silencing of gene expression^{3–5}. Here we describe a natural resistance to the DNA pararetrovirus cauliflower mosaic virus (CaMV)⁶, in non-transgenic brassicas, which also involves post-transcriptional gene silencing.

Kohlrabi (*Brassica oleracea gongylodes*) when infected by CaMV initially develops systemic symptoms from which it completely recovers by loss of virus within a matter of weeks⁷. We assessed CaMV activity in leaves during the onset of host recovery by analysing viral replication products. Cells containing a replicating virus accumulate DNA intermediates generated by reverse transcription in the cytoplasm⁸, which migrate as heterogeneous molecules on gels. They also contain supercoiled DNA, a component of the viral minichromosome in the nucleus. Between 10 and 13 days after we had inoculated plants with the virus, this pattern of replication products remained relatively unchanged. At 14–15 days after infection, a few days before amelioration of plant symptoms, there was a sudden transition in the composition of viral DNAs. Reverse transcription products were lost whereas supercoiled DNA was amplified (Fig. 1a–c).

During this transition there was a ~50-fold amplification of the viral minichromosome transcription template, but the levels of both of the main viral polyadenylated RNAs, present in infected leaves before the viral DNA transition, declined rapidly (Fig. 1d). We also observed this decline in a

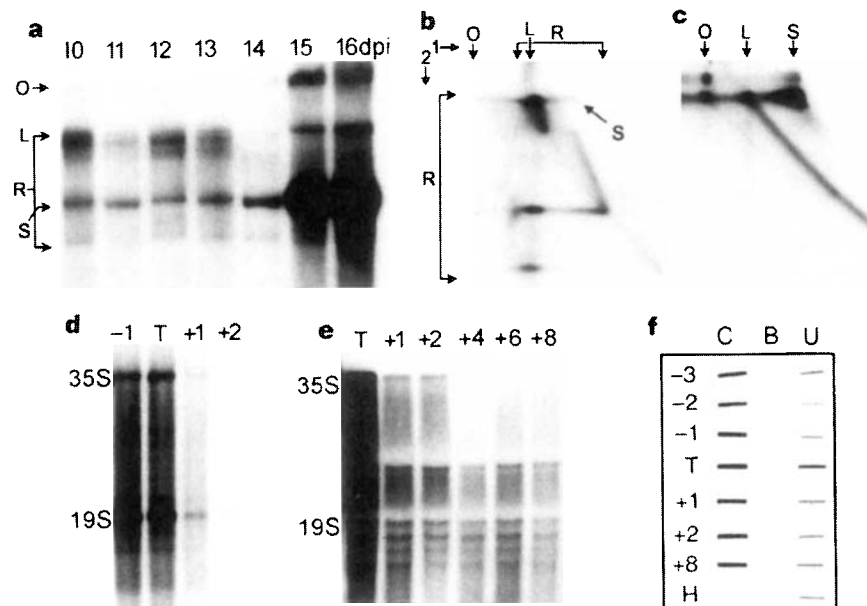


Figure 1 Changes in CaMV replication products. **a**, Time-course (days post-inoculation, dpi) of unencapsidated viral DNAs preceding host recovery, showing heterogeneous replication intermediates (R), supercoiled (S), linear (L), and open circular (O) DNA forms. **b**, 2D gel separation (first dimension (1), neutral; second dimension (2), denaturing electrophoresis) of viral DNA forms showing heterogeneous growing DNA strands (R) and a low level of supercoiled DNA (S). **c**, 2D gel separation of post-replicative viral DNA forms. **d**, Degradation of the two main CaMV polyadenylated RNAs (35S and 19S RNA) over 2 days after the transition point (T) in viral DNA forms. **e**, Total viral RNA indicating persistence of non-polyadenylated RNA fragments. **f**, Nuclear run-on transcription assay from 3 days before until 8 days after the transition point. Radioactive RNA synthesized in isolated nuclei from infected or healthy (H) plants was used to probe filters containing CaMV (C), bacterial (B) and ubiquitin (U) DNA.

total cell RNA fraction, but non-polyadenylated fragments persisted (Fig. 1e) indicating that viral polyadenylated RNAs were being targeted specifically for degradation by de-adenylation. However, neither viral RNA synthesis from the CaMV minichromosome nor that of a constitutively-expressed host gene (ubiquitin), measured by run-on transcription in isolated nuclei, showed any significant change up to 8 days after the transition (Fig. 1f). These changes in the levels of viral intermediates just before plant recovery are consistent with a sudden arrest of viral replication by gene silencing, because continued transcription seen with RNA degradation is a hallmark of post-transcriptional silencing found in a variety of guises in transgenic plants¹⁻⁵.

We detected differences in the timing of the replicative transition in different parts of the same plant, implying that the response is not elicited at the whole-plant level. As there is amplification of the CaMV minichromosome it is likely that the viral replication cycle has become deregulated, allowing retargeting of progeny viral genomes back to the nucleus. Most probably, retargeting is prevented by a viral factor synthesized during replication. The unchanged rate of transcription suggests that most of the newly amplified minichromosome DNA is inactive. We have found differences in the population of superhelical topoisomers present

but no differences in DNA methylation.

It is not clear whether DNA amplification is the cause or consequence of gene silencing. If silencing is initiated by host recognition of viral RNA as a target for degradation, then amplification of the minichromosome would result from loss of the factor preventing nuclear retargeting. Alternatively, amplification could itself initiate silencing through an ectopic interaction¹ between transcriptionally inactive and active minichromosomes. Consistent with both models, we have observed silencing of a reporter transgene with homology to CaMV RNAs but only in tissues showing DNA amplification where viral replication had ceased (unpublished results).

If, as we suggest, gene silencing underlies this natural resistance to virus infection, is antiviral defence one part of a multifaceted mechanism regulating plant gene expression, or is transgene silencing a consequence of the way that plants combat viruses? Whichever is the case, unravelling the early molecular steps should provide new opportunities for manipulating genes and controlling plant viral diseases.

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Evening carbon atom oddities

In their Scientific Correspondence, J. Sarma *et al.* (*Nature* **384**, 320; 1996) seem to show more than a simple 'even-odd carbon atom disparity'. Many of the naturally occurring organic compounds containing between 40 and 80 carbon atoms (and indeed in the frequency distribution peak of the Cambridge Structural Database; d in their figure) are undoubtedly derivatives either of hexose sugars or of aromatic rings.

The periodicity of compounds with a multiple of six carbon atoms is striking (c in their figure). In addition, many synthetic organic compounds are derived from natural (including petrochemical) sources, and therefore would reflect this natural periodicity bias in the range 6–80 carbon atoms (shown in parts a and b).

Acetate (with 2 carbon atoms), perhaps one of the prebiotic molecules synthesized from methane and formaldehyde (see S. Miller, *Science* **117**, 528; 1953) is also one of the primary building blocks of cellular organic compounds.

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Sarma *et al.* (*Nature* **384**, 320; 1996) make the interesting observation that there are more organic compounds with an even number of carbon atoms than with an odd number. The pronounced excess of C-even compounds among those with large numbers of carbon atoms could be explained if a significant proportion of the compounds are dimers or other polymers, as any polymer of a C-even compound can, of course, only be C-even, whereas polymers of C-odd compounds can be C-even or C-odd.

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Struggles to beat the system

Science and Dissent in Post-Mao China: The Politics of Knowledge

by H. Lyman Miller

University of Washington Press: 1996.

Pp. 370. \$38 (hbk), \$18.95 (pbk)

Stalinist Science

by Nikolai Krementsov

Princeton University Press: 1997. Pp. 358.

£37.50, \$45.00

David Joravsky

When China's Cultural Revolution of the 1960s 'sent down' Fang Lizhi from a physics institute to a mine, he was allowed to keep a single book, Lev Landau's treatise on field theory. Reading and re-reading it, he fell in love with general relativity and cosmology, and also with defiant liberalism. He was supposed to repent the mandarin's traditional disdain for workers and peasants, and submit to the absolute authority of the party. He became instead an unrelenting crusader for liberalism within the Communist party. He had been a member, and would join again when Deng Xiaoping reopened the universities and restored state support for scientific enquiry.

But Fang returned to theoretical physics and party politics, preaching the need for free thought in all fields and mocking the 'scientific' pretensions of political bosses who invoked Marxism as knowledge for statesmen in the same sense that physics provides knowledge for bridge-builders or biology for agronomists. Fang also mocked the crude notion of 'practicality' that the bosses brought to science policy even after they retreated from the Cultural Revolution. They favoured applied over theoretical science, within a system that tended to obstruct fruitful interaction between applied science and methods of production. Fang even preached the need for enquiry into a 'transcendent' type of belief, akin to religion, as science cannot provide the large sense of human purpose and historic direction that society requires. Marxist bosses had disgraced such inspiring vision with their scientific claims, even more than Western politicians have with talk of 'the vision thing' and vacuous religiosity.

Many ironies of this sort mark "the politics of knowledge" during the "post-Mao" phase of the Chinese Revolution, as H. Lyman Miller analyses it in his illuminating book. Xu Liangying, an older physicist than Fang — who was born in 1936 — came to a double passion for Einstein and for Mao during the heroic period of the revolution. That was when Mao was organizing a peasant army for class war as the way to liberate the country from imperialist invaders. Xu's conversion to thorough liberalism came in 1978, when he perceived that the Marxist claim of science



Trofim Lysenko: power over biologists.

requires open-ended enquiry in history and philosophy, not obedience to authority, as religious belief does.

Li Xingmin, a younger physicist than Fang, based his plea for liberalism on Robert Merton's "norms of scientific communities" — universalism, disinterestedness, organized scepticism, and 'communism' in the sense of knowledge as common property. One way or another, these Chinese physicists argued that science requires liberal democracy.

Miller tends to agree, while pointing out that many have questioned that link. His richly informative study benefited at its outset from the liberalizing concessions that China's political bosses were making — until the bloody punctuation in 1989, when Deng fell back on Mao's maxim that power comes from the barrel of a gun. Meanwhile, the Communist bosses of the Soviet Union were sliding into a confession of ideological bankruptcy, practical failure, and a statecraft of *sauve qui peut*, which would entail virtual collapse of funding for science.

Miller shows that Chinese scientists knew they were financially dependent on the authoritarian state they wished to liberalize, and vice versa: the political bosses knew, however dimly, that the practical benefits of science require a freedom of thought that undermines the authoritarian state required for modernization. Such vicious circles of mutually reinforcing dependencies and disabilities inform the sense of long-term tragedy that Miller shares with most students of Chi-

nese experience. The intelligentsia has been divided between devotion to "individual enlightenment", which seems to require liberalism in the modern age, and to "the imperatives of national salvation", which seem to require authoritarian rule. Historians of China cannot find even the fleeting moments of liberal possibility that historians of Russia love to argue about.

Miller offers comparisons with Soviet experience after 1953, when Stalin died, his lieutenants began reforms, and dissidents emerged advocating full freedom of thought. Scientists were prominent leaders in that movement, and many observers inferred the causal link that Miller endorses — science requires free thought, which entails advocacy of freedom at large. Fang Lizhi has often been called the Chinese Sakharov, and Deng's turn to repression in 1989 invites comparison with the ousting of Khrushchev in 1964, when his comrades began a 20-year effort to ignore the need for reform.

Miller is more interested in the influence of the scientific intelligentsia on the political system than in the shaping of scientists by their social context. He barely mentions, for example, the rarity of bold scientists such as Sakharov and Fang. Most scientists have been conformist, except in times of crisis when political bosses falter in their authoritarian resolve. Miller pictures the conformists as believers in the affinity of 'technocratic' science and the authoritarian state, justified by a 'totalistic' Marxism, which claims knowledge

of historical development in its totality, past, present and future. Scientists have indeed echoed such arguments, in China as well as in the bygone Soviet Union. But, after the first burst of revolutionary fervour, they sound to me like the conformists in any system, who simply 'go along to get along'. When the bosses drop "totalistic" Marxism, so do the subservient scientists.

Einstein may have been right in his gloomy observation (*The Born-Einstein Letters*; Walker, New York, 1971) that "the spinal cord plays a far more important role than the brain" in resisting the herd mentality, which is reinforced in the vast majority of scientists by "their mechanized and specialized way of thinking".

Perceptive analysis of such issues is conspicuously absent from Nikolai Kremmentsov's book, which is not only entitled *Stalinist Science*, but also claims to be an historical analysis of the entire system that shaped all the sciences. He distils the essence of the system — rule of scientists by political bosses — from the Lysenko affair, and most of the book is devoted to that imposition of pseudoscience on biology. Lev Landau and several other Nobel laureates in physics — all honoured for work done in the Stalin era — prove the rule, since they worked for Communist bosses.

Kremmentsov drags out that simplistic formula through a long book, although Soviet conformists could express it with a gesture, when asked to explain some puzzling feature of their system. They would silently point upwards, towards 'them', the unanswerable bosses. That is Kremmentsov's all-purpose scheme. When 'they' desired nuclear weapons, 'they' demanded good physics. When 'they' desired native pride during the Cold War, 'they' demanded distinctively Soviet biology, or distinctively pavlovian physiology and psychology, or Marr's school of linguistics — or anti-Marr, as Stalin suddenly decreed in a startling reversal in 1950, taking political support away from a uniquely Soviet school, and using the occasion to denounce tyranny over scholarship in any field. Such anomalies are brushed off by an explanatory scheme that nothing can disprove. What pleases the chief has the force of law. While Stalin lived, conformists said so in reverence; Kremmentsov says it in execration; the same field of force governs his mentality, with plus and minus signs reversed.

Either way, that mindset expresses avoidance and denial of painful issues in the evolving interaction of scientists and bosses. The crucial question that preoccupies Miller — to what extent the power of the bosses conflicts with the knowledge offered by scholars and suffers erosion in the conflict — hardly occurs to Kremmentsov. He ignores the most obvious test cases — economics and historiography — and denies the obvious pattern of the most notorious conflict in the natural sciences: the 'agrobiology' of Lysenko. That militant igno-

ramus turned bosses against genuine biologists in a conflict that began in agronomy and plant physiology during the collectivization of agriculture in 1929–32, then moved to plant breeding and genetics.

As Lysenko won power over research and educational institutions, genuine scientists faced choices on a scale that ran from submission through evasion to defiance, while administrators of terror were grabbing 'enemies of the people', including Nikolai Vavilov, chief of the Soviet drive to 'overtake and surpass' the US model of scientific agriculture. Conferences in 1936 and 1939 publicized the losing battle of scientific 'theory' with Lysenko's indisputable 'practice', as certified by agricultural bosses, and a 1948 conference proclaimed Lysenko's monopolistic power over biology. He got there with a long series of

He distils the essence of the system — rule of scientists by political bosses — from the Lysenko affair.

agronomic panaceas, openly scorning the tests and measurements required by genuine experts, proudly bringing into science the extreme wilfulness that Stalinist bosses had brought to peasant agriculture.

Kremmentsov simply ignores that pattern, even its grand climax in the Great Stalin Plan for the Transformation of Nature, which was announced after the 1948 conference that is the focus of his book. Massive shelter belts would make Russia's climate moist and mild, using Lysenko's method of planting trees in clusters that would need no thinning, as predicted by his denial of competition within a species. The massive failure that followed brought about a commission of inquiry in 1951, and the ecologist in charge opened the *Botanical Journal*, which he edited, to criticism of Lysenko's views on intraspecific competition.

I have been waiting to learn from post-Soviet researchers if that commission was, as I suspect, the first sign of realistic questioning within the agricultural establishment. Kremmentsov claims to have done "an exhaustive study of the archives", but that is just not so. He has ignored not only the inquiry into the tree-planting fiasco but all other sources bearing on the evolution of agricultural bosses from credence in Lysenko's panaceas through questioning to disillusion.

Kremmentsov's research concentrates on the geneticists' failing appeal to international science in 1946–48, as the Cold War replaced alliance with the West, and the Stalinist regime mounted a drive for nativistic culture at home. That was an important element in Lysenko's 1948 triumph, but Kremmentsov can hardly make it the single all-important

element simply by disregarding all else and by inflated claims of originality. He quotes Vavilov's famous line — "We will go to the stake, we will burn, but we will not renounce our convictions" — citing only an archival source, though omission of context and mistranslation are his sole originality.

Vavilov said that in a defiant speech explicitly contrasting truth by decree of the Agricultural Commissariat with truth sought by discussion among scientists. Mark Popovskii made the archival find in 1966; Zhores Medvedev quoted it at length in his landmark book of 1969, and many scholars have followed suit. K. O. Rossiianov made the major post-Soviet archival find — Stalin's editing of Lysenko's 1948 speech. Rossiianov's analysis of this document emphasizes Stalin's deletion of Lysenko's claim of class science, proletarian as against bourgeois. Communist belief in that dichotomy peaked in the early 1930s, when even mathematics was included, and declined thereafter. In 1948, where Lysenko wrote that "Every science is class science", Stalin jotted: "Ha, ha, ha! And mathematics? And Darwinism?". Kremmentsov notes that Rossiianov found the document, but ignores his analysis.

Nativistic counter-attacks on the Russian sense of provincial backwardness appeared long before the scandalous extremes of the late Stalin era, but Kremmentsov's simplistic scheme has no room for that dialectic. He says "Russian science flourished" before the Bolsheviks spoiled it. "In their discourse, self-image, and professional behaviour, pre-revolutionary Russian scientists were almost indistinguishable from their colleagues in other countries."

He does not confront the contrary opinion of Pavlov, the only Russian Nobel laureate before the physicists of the Stalin era. Pavlov lamented the rarity of good scientists, the abundant "reflex of slavery... on Russian soil". When he became a major deity of Stalinist culture, such comments were excised from his *Works*, and uncritical worship of his 'teaching' was enjoined on neuroscientists, psychiatrists and psychologists in the three 'Pavlov sessions' of 1950–52. Kremmentsov mentions only the first, and completely ignores the main issues: canonization of Pavlov's crotchety 'teaching' on the neural mechanisms of conditioning and on the correlation of mental and neural processes. Kremmentsov pretends that the heritability of conditioned reflexes was the focus. The essence of the Stalinist system must be found in Lysenko's militant ignorance, not in such genuine scientists as Pavlov and his school, struggling with the complex torments of disciplined enquiry in a 'developing country', as the euphemism of our time describes Russia and China. □

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All done by mirrors

Mirrors in Mind

by Richard Gregory

W. H. Freeman: 1997. Pp. 302. \$26.95, £25

Simon Altmann

Ever since Narcissus saw the reflection of his face on a pool of water, mirrors have fascinated humanity. There is indeed a whole world in the small mirror in Jan van Eyck's painting of the *Marriage of Arnolfini* in London's National Gallery. This is one of the many paintings involving mirrors that are discussed and beautifully illustrated in Richard Gregory's book. The use of mirrors by Renaissance architects and painters, often as aids to design, is also covered at length and, as one would expect from Gregory, numerous puzzles are discussed. Light propagation — from the old ether theories to lasers and Richard Feynman's quantum electrodynamics — is tackled with gusto, and the making and using of mirrors in science are explained and illustrated.

But what makes this book a delight to read are the short asides where Gregory displays not only the breadth of his reading but also his solid common sense. Writing about Olaüs Roëmer's discovery of a finite velocity of light, for instance, he reflects that it was far from Roëmer's intention to formulate a hypothesis and then to try to shoot it down, as by the Popperian dogma, but rather that the whole phenomenon came out as the only plausible explanation of the astronomical observations that he happened to be making.

Gregory is a master of clear and interesting presentation. At the level of this book, this is indeed very difficult to do, and it is to his great

credit that he succeeds most of the time. Here and there I would have preferred, being an impatient reader, that he had cut the Gordian knot a bit earlier by sketching what really goes on in the problem he is handling. For example, everybody knows that if you write the symbols $>C$ on a piece of paper which you then turn and view on a mirror parallel to the paper, what you see is $\supset<$. Gregory, quite rightly, points out that the mirror is symmetrical, whereas the observed reflection of the image shows that an asymmetry has been introduced that interchanges right and left. But, as Gregory correctly recognizes, Curie's symmetry principle requires that asymmetries arise from antecedent asymmetries. What is this asymmetry in the case of the mirror?

The answer to this conundrum is exceedingly easy if one is prepared to do (or to accept) a couple of lines of decent mathematics. Place the mirror vertically and call A , the reflection on the mirror and B that on a plane perpendicular to the mirror that contains its vertical axis. Call R a rotation by 180° about the mirror's vertical axis. Because you first rotate the paper and then reflect it on the mirror, you perform the operation R followed by A , and any crystallographer or group-theoretician will tell you that the product of these two operations (which can in any case be found by a very simple geometrical construction) is precisely B , the observed reflection on the vertical axis of the mirror. The asymmetrical effect noticed is merely the result of the asymmetry introduced by the rotation R . (That this is asymmetrical is clear if you take an arrow parallel to the mirror and rotate it by R .)

Of course, one man's explanation is another person's perplexity, but I find the stark truth often far more convincing than hand-waving, even if it is none other than Feynman, as quoted by Gregory, who impersonates a semaphore. I admit, however, that I was very much intrigued by all the worry that this simple question created and which is very well documented by Gregory. This example, however, illustrates the dilemma that any writer of science for the general public faces in balancing rigour and intelligibility, and anyone who could do as well as Gregory in this respect deserves praise.

In the last few chapters Gregory handles some very hard problems, from consciousness (which was imported into quantum mechanics by London and Bauer well before Wigner, as claimed here) to quantum reality, with Bohm and Bell chipping in. This is perhaps the weakest part of the book as its connection with mirrors is frankly tenuous, and as these subjects are treated at a popular level in dozens of books, it

could have been left out without detriment to the wide range covered by the author.

This is a beautifully produced book and the quality of the illustrations is enviable. Far more importantly, Gregory gets his facts right, even where they fall outside his scientific remit: he does not fall into the trap of believing the dome of St Peter's in the Vatican to be the widest in the world and he knows that this distinction goes to the earlier one at Florence. Good marks for this and for a book that many people will read with much pleasure. □

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Crunchy brains

The Neurobiology of an Insect Brain

by Malcolm Burrows

Oxford University Press: 1996. Pp. 682.

\$100, £55

Stephen J. Simpson

Fifteen years ago, neurobiology was generally seen as having failed to fulfil its earlier promise to discover the neural bases of behaviour. And this was in spite of an emphasis on apparently simple invertebrate preparations, which Graham Hoyle memorably categorized as either "squishies" or "crunchies". One of the most popular crunchy preparations has been the locust, first promoted by the influential Russian émigré biologist Boris Uvarov at London's Anti-Locust Research Centre. By the end of the 1970s there was growing disillusionment with the progress made. Kenneth Roeder, for instance, had embarked on a study of cockroach escape in the late 1940s, and still nobody understood its neural mechanisms. In 1984 Malcolm Burrows himself wrote of "aardvarkism" — "the study of yet another animal in which the pattern of movement or the way it is controlled is just a little different". Neurobiology was criticized by the ethologists as drowning in a sea of non-generalizable details.

Today, neurobiologists are generating big ideas. The nervous system is seen not as a set of fixed, dedicated circuits but as a collection of polymorphic networks, able to assume different configurations according to the dictates of chemical modulators. The cellular basis of learning is understood in principle, and its effects — the altering of synaptic strengths — are being simulated in model networks with increasingly remarkable results. Neurobiologists are even attacking the summit of consciousness, urged on by their new-found philosopher acolytes. Meanwhile, developmental neurobiologists are showing us how the nervous system builds itself from highly conserved and evolutionarily ancient genetic instructions in conjunction with its own experience. The subject is driven and pervaded throughout by new molecular techniques.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Mirror image: a detail from Jan van Eyck's *The Arnolfini Marriage*.

Burrows is an eminent and productive figure in neurobiology, and this book is an immensely authoritative treatment of locust neurobiology. His message is clear and important: while it is true that neurobiology has made huge progress, it is critical not to lose sight of the fundamental importance of detailed, experimental analyses of real nervous systems. It is because of such studies that we are where we are today. There is still much to learn, and the danger is that fashion will drive neurobiology to the point where computational and molecular approaches find themselves uncoupled from the functioning nervous system.

Burrows has chosen to concentrate on those areas of locust neurobiology where he has had greatest impact. A brief introductory chapter on locust biology is followed by chapters on the anatomy, components and development of the nervous system. He then proceeds to consider chemical communication within the nervous system before concentrating on the control of local leg movements, walking, jumping, escape, flying and breathing. Where they provide relevant data, other insects are made honorary locusts. The treatment is detailed, critical and well illustrated. Burrows points out where dogmas are unsubstantiated (one example he gives was based on a single published trace). He offers detailed assessments of the state of current knowledge, highlights lacunae and suggests areas for future research. The result is a key reference work, which should be read by all neurobiologists and behaviourists. After all, we still do not fully understand how a locust moves its leg away from a touch, let alone how a cockroach escapes. □

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Infinite possibilities

Before the Beginning: Our Universe and Others

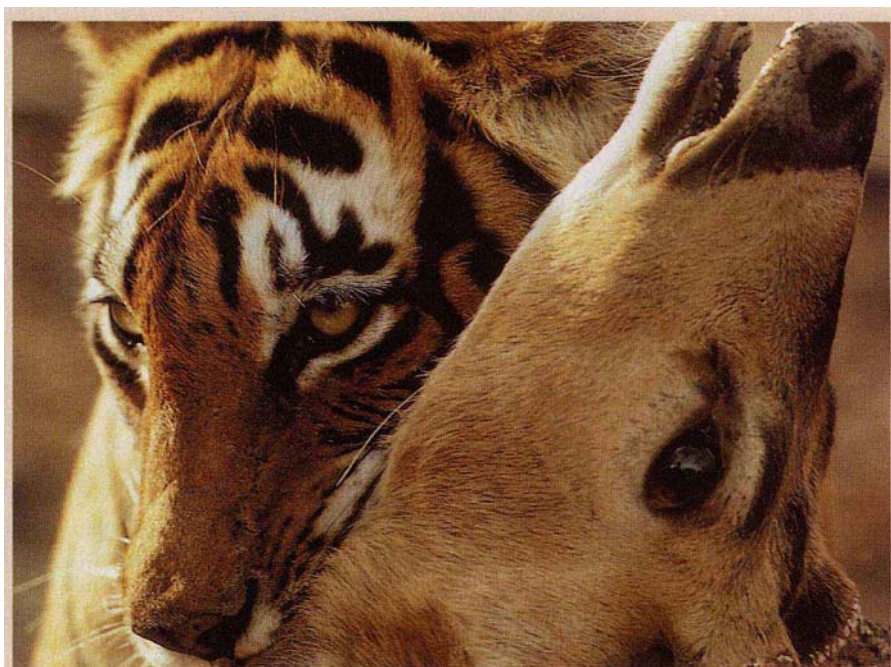
by Martin Rees

Simon & Schuster: 1997. Pp. 282. £16.99

Joseph Silk

Before the Beginning is an unusual blend of wit, asperity and cosmology, the last based around an anthropic core. The Astronomer Royal, Sir Martin Rees, takes the reader on an enthralling tour of the 'multiverse', defined by him as the ensemble of all possible universes, which he confidently asserts to be part of cosmological reality. Yet this metaphysical agglomeration of universes is inherently unobservable, because all but one, our own delicately balanced cosmos, is inimical to intelligent life.

The many-universe postulate is intellectually challenging, and purports to explain a plethora of unlikely circumstances. Why



A matter of life and death

India's tiger population, the largest in the world, is under threat from poaching. The story of a family of these magnificent animals is told in the

lavishly illustrated *A Tiger's Tale: The Indian Tiger's Struggle for Survival in the Wild* by Anup and Manoj Shah (Fountain Press, £24.95).

does the essence of life, the carbon atom, have an energy level in just the right place to enable its constituent helium atoms to be captured before they fly apart in stellar cores? Why is the vacuum empty, or almost so? Why is the nuclear force just the right strength to enable stars to form? Why is the 'weak force' as weak as it is, enabling elements to form? Why is the neutron just 14 per cent more massive than the proton, enabling hydrogen to form? Why is the electron mass only 1/1,836 of the proton mass (were it much larger, molecules such as DNA would not form)? Why were density fluctuations in the early Universe small enough, but not too small, for galaxies to form? The list seems endless.

How easy it is to postulate an infinity of universes that violate these precepts, and thereby remain unobserved, devoid of cosmologists and their books. Surely there must be some underlying theory that provides a physical explanation? Rees takes a stab at one when he eloquently describes black holes. Embedded within black holes may be other universes. A worm hole, which connects a black hole to its opposite, a white hole, would be the port of entry to a new universe that may be newly formed or ancient, and remote (or possibly not) in space and time from its parent universe. There are untold numbers of black holes in the universe. Perhaps there is an infinity of universes within universes. These may provide the anthropic framework that could explain practically all unsolved mysteries of the cosmos.

The average mind spins at this concept,

but cosmologists dauntlessly explore the consequences. The most bizarre concept is that of evolution of the baby universes: spin-off universes could display the effects of heredity and environment. Optimal procreation would require a furious proliferation of black holes, a prediction conceivably testable by X-ray astronomers.

Despite these speculative themes which permeate the book, Rees classifies himself as a reluctant conservative in his approach to science. I found myself wondering what views a radical cosmologist might espouse. Probably such a person would have included illustrations. Certainly that would lighten the text, although the author's descriptions of technical issues are noteworthy for their unusual combination of clarity and conciseness. Rees displays his political astuteness: he assesses the satellite experiment Gravity Probe-B and the cost of the Space Telescope, without seemingly taking sides. He is clearly a gambler at heart: I counted at least five bets on burning cosmological issues.

Perhaps laying odds is a subtle way of sidestepping the embarrassingly naive tendency, displayed by otherwise respectable colleagues in recent popular tracts on cosmology, to invoke theological issues. Rees is to be commended for telling the unadorned story of the latest developments in cosmology in a forthright and compelling manner. □

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Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis

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Tissue deposition of soluble proteins as amyloid fibrils underlies a range of fatal diseases. The two naturally occurring human lysozyme variants are both amyloidogenic, and are shown here to be unstable. They aggregate to form amyloid fibrils with transformation of the mainly helical native fold, observed in crystal structures, to the amyloid fibril cross- β fold. Biophysical studies suggest that partly folded intermediates are involved in fibrillogenesis, and this may be relevant to amyloidosis generally.

Tissue deposition of soluble autologous proteins as insoluble amyloid fibrils is associated with serious diseases including systemic amyloidosis, Alzheimer's disease, and transmissible spongiform encephalopathy, but the mechanisms of amyloid fibrillogenesis are poorly understood¹. Although the diverse human proteins that can form amyloid fibrils *in vivo* have unrelated sequences and tertiary folds, they can all polymerize into fibrils with similar ultrastructural appearance and identical tinctorial properties². Furthermore, the core structure of all amyloid fibrils consists of β -sheets with the strands perpendicular to the long axis of the fibre^{3,4}. Knowing which conformational rearrangements converge on the same final fold is important for understanding the determinants of protein structure, and may enable the development of rational approaches to the treatment of amyloid diseases. However, although a lot is known about the mutations and substitutions responsible for hereditary and acquired amyloidosis (see, for example, refs 5–14), there is little detailed information about the relationship between structure and folding in amyloid proteins.

The two known natural mutations in the human lysozyme gene both cause autosomal dominant hereditary amyloidosis¹⁵. Affected individuals are heterozygous for single base changes which encode non-conservative amino-acid substitutions, Ile56Thr and Asp67His, respectively, and the amyloid fibrils consist exclusively of the variant protein¹⁵ (see below). The structure, dynamics and folding of c-type lysozymes and the related α -lactalbumins have been studied comprehensively^{16–23}. The identification of lysozyme as an amyloidogenic protein was therefore of particular interest.

Here we present a detailed analysis of the structure, stability, conformational dynamics and fibrillogenic properties of the amyloidogenic lysozyme variants which links the formation of amyloid with the folding behaviour of proteins.

Amyloidogenic variants have native folds

Wild-type lysozyme and the amyloidogenic variants¹⁵, which have

Table 1 Molecular masses and enzyme characteristics of wild-type and variant lysozymes

Protein	Electrospray ionization mass spectrometry		Enzyme properties	
	Observed M_r	Predicted M_r	K_M (μ M)	k_{cat} ($M s^{-1}$)
Natural wild type	14,691	14,693	not done	not done
Recombinant wild type	14,696	14,693	16.5 (4)	14.5 (0.5)
Recombinant Ile56Thr	14,681	14,680	18.5 (3)	15.0 (0.5)
Recombinant Asp67His	14,718	14,715	38.0 (9)	9.5 (0.5)

K_M and k_{cat} are given as mean (s.d.).

not previously been isolated, were produced in the baculovirus expression system, and the correct mass of each purified, recombinant protein was demonstrated by electrospray ionization mass spectrometry²⁴ (ESI-MS) (Table 1). They were all enzymatically active, although the Asp67His variant had a higher K_M and lower k_{cat} than the wild type and the Ile56Thr variant (Table 1).

The native folds of the two amyloidogenic variants determined by X-ray crystallography both resemble that of the wild-type protein¹⁸ (Fig. 1a), and all have the four correct, intact disulphide bonds. However, substitution of Asp 67 by histidine destroys the network of hydrogen bonds that stabilizes the β -domain, resulting in a large, concerted movement of the β -sheet and the long loop within the β -domain away from each other, distortion of the active site, and an overall displacement of backbone atoms in the vicinity of residues 48 and 70 by as much as 11 Å (Fig. 1a). The crystal structure of the Ile56Thr variant does not show such changes, indicating that the movements in the β -domain are not the direct cause of amyloidogenicity. However, closer inspection of the two structures demonstrates that subtle, but structurally significant, changes at the interface region between the α - and β -domains occur in both variants (Fig. 1b, c). Ile 56 is a pivotal residue for the structural integrity of the lysozyme fold in that it links the two domains; its importance is emphasized by its high conservation in the lysozyme sequences¹⁹. An increased B-factor (11.7 Å²) was found for the C α

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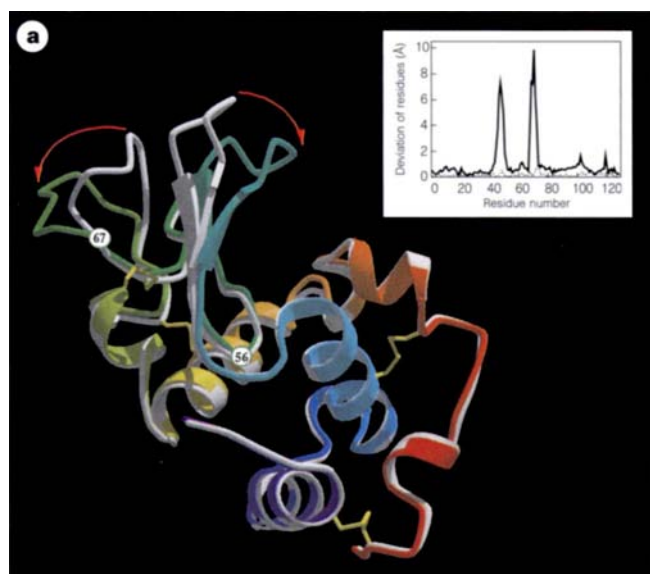
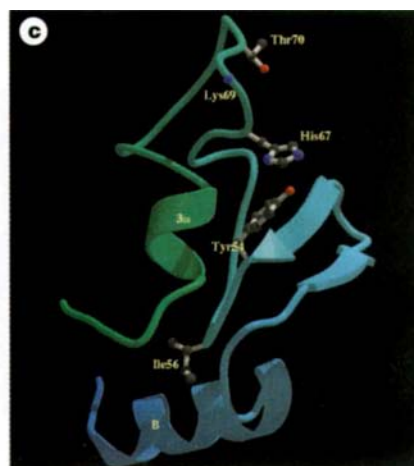
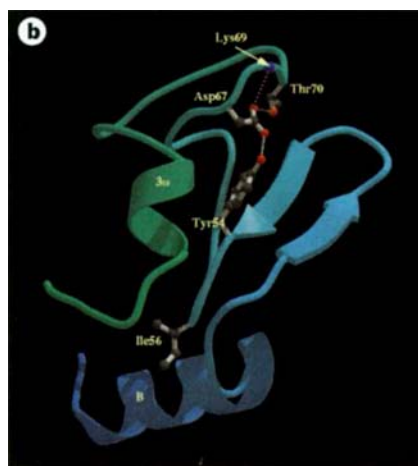


Figure 1 a, Overlay of ribbon diagrams representing the structures of wild-type human lysozyme (grey) and the soluble form of Asp67His lysozyme (coloured from blue at the N terminus to red at the C terminus). Red arrows indicate the relative movement in the positions of residues 45–54 and 67–75 in the Asp67His variant compared with those in the wild-type protein. The four native disulphide bonds in the wild-type protein and both variant structures are shown in yellow. Inset, plot showing the displacement of the residues of the Asp67His (solid line) and Ile56Thr (broken line) variants from their positions in the wild-type protein. **b**, Ribbon diagram of the β -domain of the wild-type protein, showing the critical role of Asp 67 in the network of hydrogen bonds that stabilizes this domain. **c**, Ribbon diagram illustrating the same region in the Asp67His variant and the disruption of the domain that occurs when the aspartate at position 67 is replaced by histidine and the hydrogen-bonding network is destroyed.



atom of residue 56 in the Ile56Thr variant, relative to that of Ile 56 in the wild-type protein ($6.4 \text{ \AA}^2$). This is presumably due to the now hydrophilic side chain being in an unfavourable hydrophobic environment, even though its hydrogen-bonding potential seems to be at least partly satisfied by a hydrogen bond (length $3.3 \text{ \AA}$) to one of the water molecules found in both the wild-type and the variant structures. In the Asp67His variant, the changes in the conformations of the β -sheet and long loop are transmitted down to residue 56, resulting in a new orientation for the side chain and an increase in the B-factor of the C α atom of the latter ($9.7 \text{ \AA}^2$). This suggests that the crucial interface region between the α - and β -domains is less constrained in both variants than in the wild-type protein. This common feature of both structures implies that it could be an important factor in their amyloidogenic properties. Substitution of Ile 55 in hen lysozyme (the corresponding residue to Ile 56 in human lysozyme) by threonine also reduces the stability of the protein and generates a tendency to aggregate²⁵, further supporting the view that this residue is essential for the maintenance of the lysozyme fold.

Fibril formation occurs *in vitro*

In contrast to the reversible thermal denaturation of wild-type lysozyme from both natural and recombinant sources, the amyloidogenic variants were inactivated by heating (Fig. 2). The variants were also less stable than wild-type lysozyme, with unfolding transition midpoints reproducibly 10°C or more below that of

the wild-type protein. Furthermore, both variants eventually lost all activity when incubated at pH 7.4 at the physiological temperature of 37°C , whereas the wild-type protein retained full activity under these conditions (data not shown).

The amyloidogenic lysozyme variants also aggregated on heating, unlike the wild-type protein. The rate and extent of aggregation varied with protein concentration and the expression batch, and although the aggregates stained with Congo red²⁶, they generally did not give the green–red birefringence in polarized light that is pathognomonic of amyloid. Nevertheless, negatively stained electron micrographs revealed rigid, non-branching fibres of indeterminate length and approximately 8–10 nm diameter, with the typical appearance of amyloid fibrils. Fibrils were also seen in electron micrographs of the sediment that formed spontaneously at 4°C in concentrated solutions of both Ile56Thr and Asp67His variants (Fig. 3). Strikingly, one preparation of heated Asp67His lysozyme contained fibres that stained with Congo red and did display the diagnostic green birefringence, confirming the capacity of the variant to form the classical amyloid structure *in vitro* independently of any other component.

Fourier-transform infrared spectroscopy (FTIR) of recombinant Asp67His lysozyme heated under conditions in which fibrils form demonstrated a predominance of β -structure and a loss of helical structure relative to the wild-type protein (Fig. 4). The FTIR spectrum also indicated the persistence of some helical structure in the heated sample. This could arise from residual soluble forms of

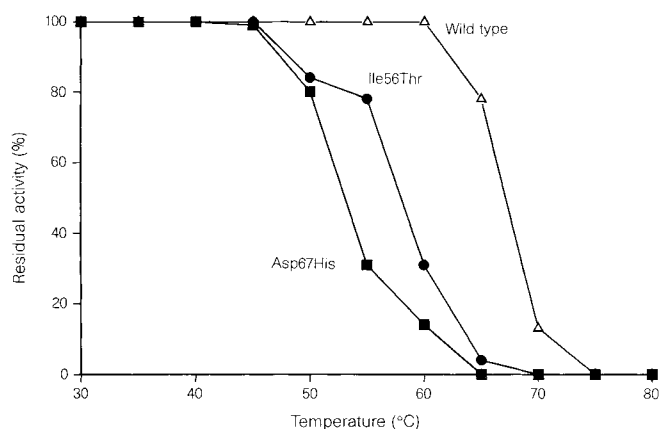


Figure 2 Melting temperatures of wild-type and amyloidogenic variant lysozymes.



Figure 3 Electron micrograph of sediment from the Ile56Thr lysozyme, after standing at 1 mg ml⁻¹ for 14 days at 4°C in 10 mM HEPES, 1M LiCl, pH 8.0. Scale bar, 100 nm.

Table 2 Recovery of enzymatically active lysozyme from *ex vivo* Asp67His lysozyme amyloid fibrils

	<i>V_e</i> on gel filtration (ml)	Lysozyme recovered (μg)		Electrospray ionization mass spectrometry	
		Protein by A ₂₈₀	Active enzyme	Observed <i>M_r</i>	Predicted <i>M_r</i>
<i>Ex vivo</i> Asp67His lysozyme fibrils solubilized in 6 M guanidinium-HCl, pH 6.7	13.20 (0.13)	100	80	14,716.0 14,733.8	14,715 for Asp67His lysozyme 14,729 for Asp67His lysozyme with MetSO
<i>Ex vivo</i> Asp67His lysozyme fibrils solubilized in 6 M guanidinium-HCl, pH 6.7, 0.1% 2-mercaptoethanol	12.57 (0.06)	113	0	No signal obtained	

V_e given as mean (s.d.) from 3 experiments.

the lysozyme variant, from persistence of helical structure in the fibril, or both.

Fibrillogenesis is reversible

Our identification of a second Asp67His family²⁷, apparently unrelated to the original kindred¹⁵, provided the opportunity to study Asp67His lysozyme amyloid fibrils. The X-ray fibre diffraction pattern (not shown) contains distinctive reflections at 4.6–4.8 Å on the meridian and at 8–14 Å on the equator of the image, indicating that the underlying ordered structure is a β-sheet in which the constituent β-strands are at right angles to the fibre axis¹. Such cross-β structures are characteristic of amyloid and have also been described in the glutamine repeats that are associated with several neurodegenerative diseases, including Huntington's disease, and which cause oligomerization of proteins²⁸. The fibre diffraction pattern of *ex vivo* Asp67His lysozyme fibrils contains no reflections attributable to helical structure, suggesting that, if helices persist after transformation of the soluble protein to the fibrillar form, they are not regularly ordered.

As previously reported for Ile56Thr fibrils¹⁵, 85% of the total protein in water-extracted²⁹ Asp67His fibrils ran in reduced SDS-PAGE in the same position as intact monomeric lysozyme; the remainder consisted of oligomeric lysozyme aggregates and traces of uncharacterized high-molecular-weight material that is seen in all *ex vivo* amyloid fibrils. However, like the fibrils from the Ile56Thr case¹⁵, the Asp67His lysozyme fibrils could not be dissociated to a form detectable by ESI-MS using either acetonitrile/acetic acid mixtures or up to 100% formic acid. We therefore solubilized some of the *ex vivo* Asp67His fibrils by denaturation in 6 M guanidine HCl, isolated the lysozyme by gel filtration in the same denaturing conditions, and attempted to refold it by dialysis into water at pH

3.8, a solvent in which natural wild-type lysozyme is stable. Although some reaggregation occurred during dialysis, the recovered lysozyme was detectable by ESI-MS with a mass corresponding to intact, monomeric, Asp67His variant (Table 2). The exclusive presence of variant lysozyme in either Asp67His or Ile56Thr (ref. 15) *ex vivo* amyloid fibrils indicates that their pathological aggregation does not engage wild-type lysozyme *in vivo*, presumably because the wild type has greater stability than the variants.

Remarkably, the refolded Asp67His variant lysozyme was enzymatically active, in contrast to the absence of any activity in the original fibril preparation, using soluble penta-*N*-acetyl-β-chitopaentaoiside substrate. Although the variant protein must have undergone major conformational change *in vivo* to form characteristic cross-β amyloid fibrils, it remained able, after unfolding, to renature spontaneously into the active enzyme. However, when the disulphide bonds within the chain were reduced with 2-mercaptoethanol during solubilization and unfolding, no lysozyme activity was observed and the protein could not be detected by ESI-MS (Table 2).

Stabilized molten globule intermediate

We have used circular dichroism to monitor the unfolding behaviour of the two lysozyme variants under conditions in which they form fibrils *in vitro*. The results (Fig. 5a–d) showed that both variants were less thermostable than the wild-type protein, with midpoints of denaturation approximately 12°C lower than that of the wild-type protein at pH 5.0. More importantly, however, the unfolding transition of the two amyloidogenic variants, although reversible under conditions in which fibril formation did not occur, was not cooperative. This resulted in a partly folded state being significantly populated near the midpoint of unfolding. This state

Table 3 Crystallographic data statistics

Parameters	Recombinant wild type	Asp67His variant	Ile56Thr variant
Structure determination			
Resolution (Å)	30–1.8 Å	30–1.75 Å	30–1.8 Å
Data completeness (%)	89.9	90.9	92.8
I/σ for all hkl	12.1	8.5	11.2
I/σ at high-resolution limit	3.7	3.1	3.6
Observations	51,285	41,333	49,524
Unique reflections	10,221	11,071	10,578
Space group	$P2_12_12_1$	$P2_1$	$P2_12_12_1$
Unit cell (Å)	$56.62 \times 60.88 \times 33.79$	$37.34 \times 31.86 \times 51.50$	$56.80 \times 60.89 \times 33.70$
β -angle		102.56°	
R_{sym} (%)	8.3	10.3	9.2
Structure refinement			
Resolution (Å)	8–1.8 Å	8–1.8 Å	8–1.8 Å
Non-H atoms	1,029	1,031	1,028
Water molecules	38	115	43
R -factor (reflections)	21.3 (10,216)	22.8 (10,216)	21.1 (10,513)
Average B -value	13.11	13.54	16.76
R.m.s.d. bond lengths (Å)	0.013	0.015	0.013
R.m.s.d. bond angles (°)	1.674	1.814	1.643
R.m.s.d. dihedral angles (°)	24.132	23.076	24.601
R.m.s.d. improper angles (°)	1.463	1.666	1.391

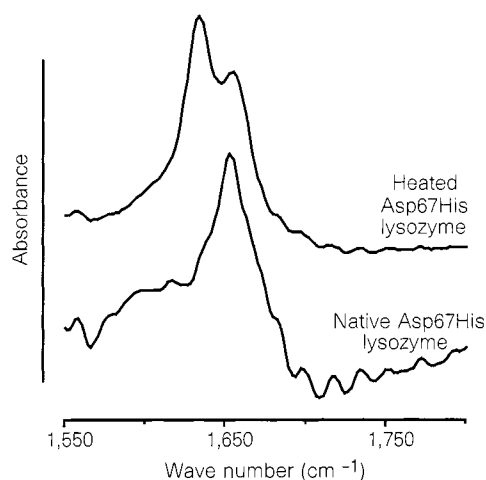


Figure 4 FTIR spectra, offset for comparison, of soluble Asp67His lysozyme, and after heating to induce fibril formation. The dominant absorption band centred at $1,655\text{ cm}^{-1}$ in the untreated sample reflects the large α -helical component in the soluble, native protein. The shift to absorption at about $1,630\text{ cm}^{-1}$ after heating indicates an increase in β -sheet content. The shoulder at $1,655\text{ cm}^{-1}$ in the heated sample demonstrates the persistence of some helix.

had substantial helical secondary structure but lacked persistent tertiary interactions (Fig. 5b, d). Such behaviour is quite different from the cooperative unfolding displayed by the wild-type protein under these conditions (Fig. 5a, c), but is similar to the thermal unfolding of wild-type human lysozyme under conditions of extremely low pH, where the protein unfolds through a partially structured intermediate with circular-dichroism properties similar to those identified here for the variants at pH 5.0 (ref. 30). Moreover, species with similar properties have been identified on the kinetic or equilibrium folding pathways of other lysozymes and α -lactalbumins^{21,23}. At the midpoint of thermal denaturation, the partly folded amyloidogenic intermediates bound the hydrophobic dye 1-anilino-naphthalenesulphonic acid (ANS) (Fig. 5f); this is one of the major characteristics of the previously characterized lysozyme and α -lactalbumin molten globules^{22,23}. The Ile56Thr variant also bound ANS at 20 °C, although it generated weaker

fluorescence than at its midpoint of unfolding, indicating the presence of exposed hydrophobic regions even at this temperature.

Transient unfolding

The conformational dynamics of the wild-type and variant proteins in solution at 37 °C were investigated by using ESI–MS to monitor the exchange of the labile amide and side-chain hydrogens with solvent deuterons (Fig. 6). The hydrogen exchange kinetics of the two amyloidogenic variants were remarkable in that there was very little protection from exchange (Fig. 6); in contrast, about 55 hydrogens were strongly protected from exchange in the wild-type protein under these conditions (Fig. 6). The lack of protection of the variants cannot be explained simply by their thermal destabilization relative to the wild-type protein; a chemically modified hen lysozyme, which lacks a single disulphide bridge, has a midpoint of unfolding 24 °C lower than the wild-type protein, but still shows significant protection against hydrogen exchange³². Rather, these results suggest that the alterations in the domain interface of the Ile56Thr and Asp67His variants reduce the stability and cooperativity of the native fold such that both the amplitude and frequency of the native-state fluctuations are increased, even at 37 °C, to an extent that allows solvent water access to the interior of the protein. The degree of protection of the variants is similar to that previously observed by ESI–MS in the well-characterized, partly folded, molten globule state of α -lactalbumin³³. We suggest, therefore, that the aggregation-prone, partly folded forms are present in dynamic equilibrium with the native protein at significant concentrations, even under conditions where the native state is thermodynamically stable, and could be important determinants of the amyloidogenic properties of the variants and the slow deposition of fibrils observed at 4 °C.

The previously characterized kinetic and equilibrium partly folded intermediates of lysozymes and the α -lactalbumins all have persistent structure in the α -domain but lack stable, native-like structure in the β -domain^{20,21,31}. Based on the present results, we propose a model for lysozyme fibrillogenesis in which association of the partly folded forms of the variants occurs through the unstable β -domain (Fig. 7). In support of this, a peptide corresponding to the β -sheet region of hen lysozyme has been shown to form extensive intermolecular β -structure³⁴. The development of stable β -structure through such intermolecular association could then act as a template for the progressive recruitment of polypeptide chain into the nascent fibril, with the growth of hydrogen-bonded β -structure providing the context³⁵ for the deposition of poly-

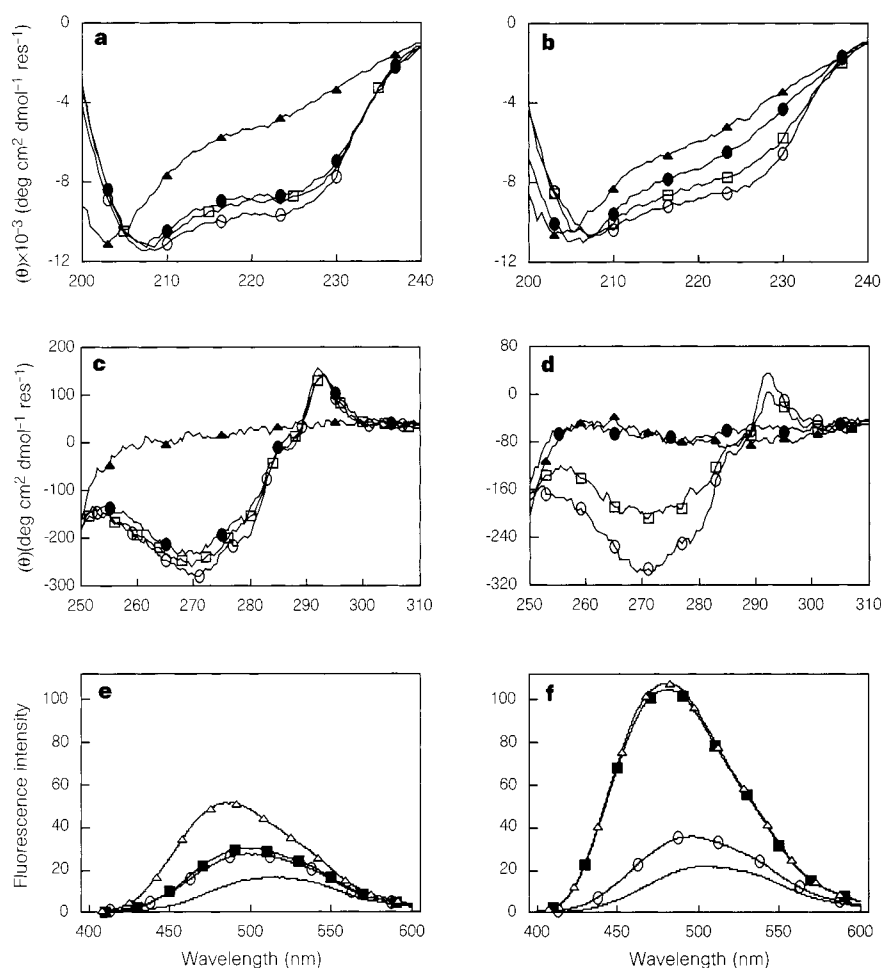


Figure 5 Thermal denaturation of wild-type and Asp67His human lysozymes. Far-UV (a) and near-UV (c) CD spectra for wild-type human lysozyme and far-UV (b) and near-UV (d) CD spectra for the Asp67His variant lysozyme, all obtained in water at pH 5.0 and collected at 20 (○), 60 (□), 70 (●) and 95 °C (▲). Binding of 1-anilino-naphthalenesulphonic acid (ANS) to the proteins at 20 °C (e) and at the

midpoint of thermal denaturation, T_m (f). The midpoint of thermal denaturation was 74 °C for wild-type human lysozyme and 62 °C for the Asp67His and Ile56Thr variants. Fluorescence intensity (arbitrary units) is shown for ANS-containing buffer solution (solid line), wild-type lysozyme (○), Asp67His lysozyme (■) and Ile56Thr lysozyme (Δ). (θ), molar ellipticity.

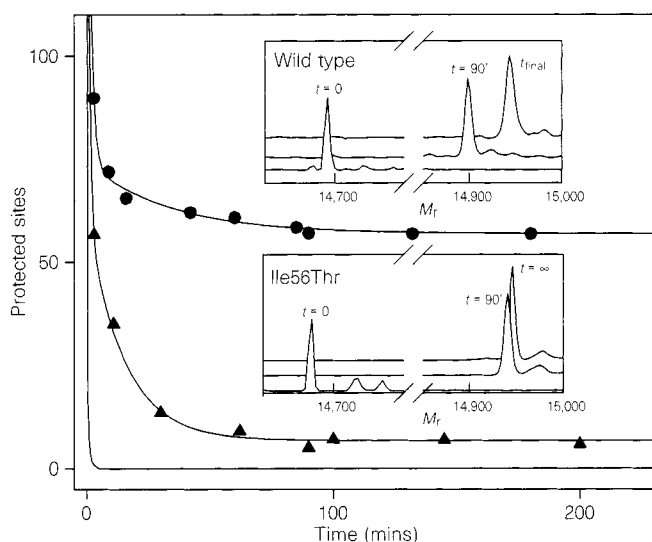


Figure 6 Kinetic profiles of hydrogen exchange at pH 5.0, 37 °C, for wild-type human lysozyme (circles) and Ile56Thr variant (triangles) monitored by ESI-MS. The exchange profile for the Asp67His variant is very similar to that shown here for the Ile56Thr variant. The plain black line is the simulated curve predicted⁴⁸ for a completely unstructured peptide with the sequence of human lysozyme at pH 5.0 and 37 °C. The two inserts represent mass spectra obtained for the wild-type and variant protein before exposure to D₂O ($t=0$), 90 min after the initiation of exchange ($t=90'$), and after heating to 70 °C for 15 min to facilitate complete exchange (t_{final}). Data were corrected for the residual 10% H₂O.

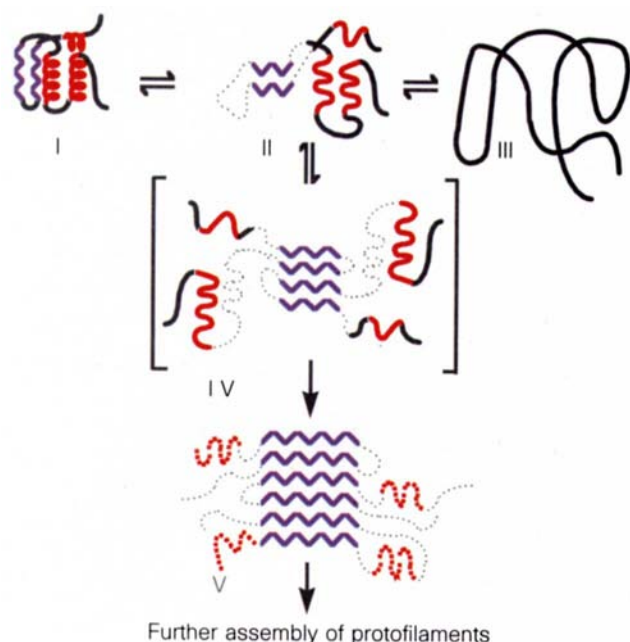


Figure 7 Proposed mechanism for lysozyme amyloid fibril formation. Blue, β -sheet structure; red, helical structure; dotted lines, undefined structure. A partly folded, molten globule-like form of the protein (II), distinct from the native (I) and denatured (III) states, self-associates through the β -domain (IV) to initiate fibril formation. This provides the template for further deposition of protein and for the development of the stable, mainly β -sheet, core structure of the fibril (V). The undefined regions in V represent the possibility that not all of the polypeptide sequence is involved in the cross- β structure. The nature of this residual structure in V is not known, and the figure is not intended to represent any defined secondary structural type (see text).

peptide chain in the stable cross- β fold. The FTIR data indicate that fibrillogenesis involves an increase in β -sheet structure; conversion of α - to β -structure will be easier in the molten globule state than the native state because of the much lower cooperativity of the unfolding process²³.

Mechanism of amyloid fibril formation

All amyloid fibrils have similar morphological and tinctorial characteristics and are predominantly β -sheet structures, indicating that a conformational change, involving a helix-to-sheet transition in some proteins, occurs during fibril formation¹³. As in lysozyme amyloidosis, amino-acid substitutions responsible for amyloid formation in immunoglobulin light chain⁸ and transthyretin variants¹¹ affect the stability of the proteins and their tendency to aggregate. It has been suggested that molten globule states are critical in protein folding and related structural transitions^{36,37}. We propose that transient population of the amyloidogenic proteins in a molten globule-like state that lacks global cooperativity is an important feature of the conversion from the soluble to the fibrillar form. The structure of a domain of the prion protein PrP(121–231)³⁸ also demonstrates that residues for which mutations are associated with prion disease are involved in maintenance of the hydrophobic core. It has also been suggested that the conversion of the cellular form to the infectious form, which involves helix-to-sheet conversion, may be initiated by the β -sheet elements of the native structure³⁸. There is no evidence for infectivity of other types of amyloidosis or for conversion of non-amyloidogenic wild-type proteins by exposure to amyloidogenic variants. Nevertheless, the mechanism we have described for lysozyme amyloidosis (Fig. 7), proceeding from the soluble forms of amyloidogenic precursor

proteins through a transient population of intermediates with the structural characteristics of molten globules, and on to intermolecular β -sheet association, may occur generally in the amyloidoses. *Note added in proof:* After submission of this manuscript Funahashi *et al.*⁴⁹ reported that the crystal structure of Ile56Thr variant lysozyme is similar to that of wild-type human lysozyme, as shown here. Their physicochemical studies also demonstrate reduced protein stability and altered folding kinetics, strongly supporting the idea that partly folded intermediates play an important role in lysozyme fibril formation. □

Methods

Lysozyme expression. Human wild-type and Asp67His variant lysozyme cDNAs were amplified from macrophage RNA of the Asp67His proband. The 5' primer, CTTGGATCCCTAGGACTCTGACCTAGCAGT, contained a *Bam*HI site and targeted sequence in the untranslated region of the cDNA. The 3' primer, NNNNNNTCTAGATTACACTCCACAACCTTG, contained an *Xba*I site and 6 random nucleotides at its 5' end to facilitate cleavage³⁹. The Ile56Thr variant sequence was obtained by *in vitro* mutagenesis of wild-type cDNA (pAlter system, Promega). The three cDNAs were cloned into the *Bam*HI/*Xba*I sites of pBacPAK8, transfected into Sf9 cells, and recombinant baculoviruses were selected and amplified (Clontech). Lysozyme was detected⁴⁰ in medium from infected cells; spinner cultures of Hi5 and Sf9 cells, infected at multiplicity of infections from 1 to 15, yielded 2–20 mg l⁻¹.

Isolation and characterization of recombinant lysozymes. Lysozymes were isolated by cation-exchange chromatography (Macrorep S, BioRad) and FPLC gel filtration (Superose 12, Pharmacia), and gave single bands on reduced SDS 8–18% gradient PAGE (Pharmacia ExcelGel) stained with silver. Lysozyme enzyme kinetics were determined with penta-*N*-acetyl- β -chitopentaoside⁴¹. For electron microscopy, protein diluted in water was placed on a formvar-coated grid and negatively stained with 2% sodium phosphotungstate.

Crystal-structure determination. All crystals were grown by vapour diffusion at 20 °C; wild-type lysozyme from 30 mM sodium phosphate, 2.5 M NaCl, pH 4.9, Asp67His from 0.1 M ammonium sulphate, 30% PEG 8000 and Ile56Thr from 0.16 M ammonium sulphate, 24% PEG 8000. Drops initially contained equal volumes of protein (10 mg ml⁻¹ in 10 mM HEPES, 0.4–0.5 M LiCl, pH 7.1–8.0) and reservoir buffer. X-ray data were collected at 15 °C using a MAR RESEARCH image plate, mounted on a Rigaku rotating anode X-ray generator. Data processing and reduction were performed with the DENZO and SCALEPACK programs⁴². The CCP4 suite of programs⁴³ was used for map calculation and coordinate analysis. The structures were refined using cycles of restrained molecular dynamics and positional refinement in X-PLOR 3.1 (ref. 44) and manual fitting with the interactive graphics programme O⁴⁵. Crystallographic data statistics are given in Table 3. Recombinant wild-type human lysozyme data were collected as a control set. The initial $2F_o - F_c$ map for the Ile56Thr variant structure was produced with phases calculated from the refined model of recombinant wild-type human lysozyme¹⁸. The structure of Asp67His lysozyme was solved by molecular replacement using X-PLOR⁴⁴. The solution indicated changes in the conformation of the β -domain (regions 45–54 and 66–75). The structure was rebuilt manually and refined with cycles of molecular dynamics and model building guided by interpretation of electron density maps. Figures were generated with O⁴⁵ and a version of MOLSCRIPT⁴⁶ modified by R. Esnouf.

Thermal stability. Melting temperatures were determined by placing wild-type and variant lysozymes at 2.5 μ g ml⁻¹ in 20 mM HEPES, 100 mM LiCl, pH 7.23, containing 1% (w/v) bovine serum albumin, for 15 min at the temperatures shown, then cooling to 21 °C for 15 min, before enzyme assay⁴⁷ at 21 °C.

Infrared spectroscopy. Infrared spectra of proteins (~5 mg ml⁻¹) dissolved in D₂O buffer containing 20 mM Tris-HCl, pH 7.0 (uncorrected for deuterium effects) were collected (Bruker IFS-55 spectrometer, 2 cm⁻¹ resolution) before and after heating to induce unfolding and fibril formation.

Recovery of active lysozyme from *ex vivo* lysozyme amyloid fibrils. Amyloid fibrils were isolated by water extraction from amyloidotic liver tissue of a patient with the Asp67His lysozyme gene mutation who underwent emergency liver transplantation following spontaneous rupture of the liver. Lyophilized fibrils were incubated for 72 h in 6 M guanidine HCl, pH 6.7, with or without 0.1% (w/v) 2-mercaptoethanol, then centrifuged to remove

insoluble material. The supernatants were fractionated by FPLC gel filtration (Superose 12, Pharmacia) eluted with the corresponding solvent; the main peak in each case, composed of monomeric lysozyme, was pooled and dialysed against H₂O, pH 3.8. Lysozyme enzyme activity was quantified⁴⁰, and the molecular mass of the solubilised protein was determined by ESI-MS²⁴. The elution volume (V_e) of lysozyme recovered in the presence of mercaptoethanol was significantly lower than that obtained without reduction, corresponding to the expected larger volume of unfolded lysozyme without disulphide bridges, confirming that reduction had occurred. This material did not recover enzyme activity and gave no signal in the mass spectrometer, presumably because of its propensity to aggregate. Natural *ex vivo* wild-type lysozyme dissolved in 6 M guanidine-HCl, pH 6.7, and run on the same column as a control, eluted with the same V_e as the Asp67His lysozyme from the amyloid fibrils.

Circular dichroism. Spectra were collected at 1-nm intervals using a JASCO J720 spectropolarimeter with 1 mm and 10 mm path-length quartz cuvettes in a temperature-controlled housing, over the wavelength ranges 190–250 nm and 250–360 nm respectively. The protein samples were at 0.22 mg ml⁻¹ in H₂O, pH 5.0, based on $E_{280}^{1\%}$ for 1 cm path length = 25.5.

ANS binding and fluorescence. Proteins at 0.2 mg ml⁻¹ in 50 mM sodium acetate, pH 5.0, with final ANS concentration 0.1 mg ml⁻¹, were analysed in a Perkin Elmer luminescence spectrometer LS50B with a temperature-controlled cell; excitation wavelength, 385 nm; total fluorescence emission monitored between 400 and 600 nm.

Deuterium exchange and mass spectrometry. Proteins were washed extensively in water, pH 3.8, then equilibrated in water, pH 5.0, at 20 μ M for determination of mass spectra³³. Hydrogen exchange was initiated by a 10-fold dilution from protein in H₂O at pH 5.0 and 37°C into D₂O at pH 5.0 (uncorrected for deuterium effects) and 37°C.

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Correspondence should be addressed to M.B.P. (e-mail: mpepys@rmps.ac.uk). Requests for molecular reagents should be addressed to D.R.B. (e-mail: dbooth@rmps.ac.uk) and for biophysical data to M.S. (e-mail: margie@bioch.ox.ac.uk). The variant-lysozyme coordinates have been deposited in the Protein Data Bank, accession no. 1LOZ for Ile56Thr and 1LYY for Asp67His.

Absence of a planetary signature in the spectra of the star 51 Pegasi

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51 Pegasi, one of many nearby Sun-like stars, was undistinguished until the recent detections of apparent variations in its radial velocity, which have been attributed to reflex motion caused by a planetary companion^{1,2}. The velocity variation inferred from variations in the spectral lines of 51 Peg has an amplitude of $56\text{--}59\text{ m s}^{-1}$ and a period of 4.23 days, implying a planet of at least half the mass of Jupiter moving in an embarrassingly small orbit of 0.05 astronomical units. But the techniques currently used to identify these exceedingly small radial velocity variations do not allow for the possibility that changes of comparable size might be occurring in the intrinsic shapes of the spectral lines; such variations are expected when a star pulsates or has spots on its surface, and could be mistaken for radial velocity variations. Here I present high-spectral-resolution observations of 51 Peg that show that its spectral lines exhibit intrinsic shape variations with a period of 4.23 days, and an amplitude comparable to that previously attributed^{1,2} to radial velocity variations. As the presence of a planet will not influence the shapes of spectral lines, these variations are likely to reflect a hitherto unknown mode of stellar oscillation. The presence of a planet is not required to explain the data.

As part of a long-term study of magnetic-cycle-type variations in cool stars^{3,4}, observations of 51 Peg have been collected for a number of years at the University of Western Ontario using a coude spectrograph⁵ having a resolving power of $\sim 100,000$. This Letter is based on data from 39 exposures taken between 1989 and 1996.

Two types of information were extracted from the spectra. First, the asymmetry of the $6,252.57\text{-}\text{\AA}$ Fe I spectral line was measured. Spectral lines in cool stars are asymmetric primarily because of granulation on their surfaces⁶⁻⁸. This asymmetry has been specified using The line bisector, that is, the locus of points bisecting horizontal line segments running between the sides of the spectral line. The line bisectors for cool stars have a somewhat distorted 'C' shape, the details of which vary from one spectral line to the next. Systematic changes in bisectors occur with effective temperature and luminosity of the star⁹. Non-radial pulsation¹⁰ and rotational modulation arising from a non-uniform surface¹¹ can cause temporal variations in the asymmetry. Figure 1 illustrates the 39-exposure average line bisector for 51 Peg. The velocity span, measured between bisector points at 0.50 and 0.90 of the continuum (shown by horizontal arrows Fig. 1) is a simple measure of the overall slope of the bisector. The changes in the line profiles are subtle and not at all apparent from casual inspection of the spectra; the spectral lines are $\sim 15,000\text{ m s}^{-1}$ wide, against which the bisector span ($\sim 50\text{ m s}^{-1}$) and its variations are minuscule.

The second parameter measured is the ratio of the central depths of two lines having different sensitivity to Doppler broadening and to temperature, $6,251.83\text{-}\text{\AA}$ V I to $6,252.57\text{-}\text{\AA}$ Fe I. Radial velocities, in the sense of displacement of the whole spectral line, are not available from these data because no absolute velocity reference frame is recorded during the observations.

Variations occur in both the line bisector and the line-depth ratio. Although the dates of most exposures are too far apart to allow identification of periods as short as a few days, we can use the orbital parameters obtained by Mayor and Queloz¹ (a period of 4.2293 d

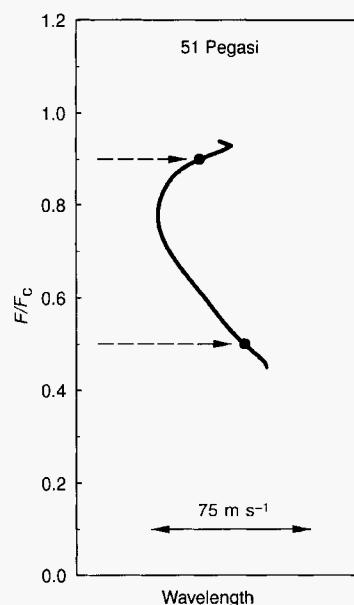


Figure 1 The average of 39 exposures of the line bisector of Fe I $6,253\text{ \AA}$. The plot of F/F_c , flux normalized to the continuum, against wavelength displays the typical 'C' shape induced by granulation on the surface of the star. Hot convective cells rise, giving to their light a net Doppler shift to the blue (integrated over the hemisphere of the star facing us), while cooled material returns downward, giving a net Doppler shift to the red. As the fraction of light coming from the hot material is substantially larger than the light from the cooled material, the combined effect is an asymmetrical broadening of the spectral lines. The wavelength difference, expressed in velocity units, between the two indicated points is the velocity span used to parametrize the cyclic variations in the upper panels of Fig. 2.

and an epoch of Julian day 2449797.773) to compute a phase diagram for the spectroscopic data. The phase was computed for each exposure, and individual values of velocity span and line-depth ratio were averaged in phase bins according to the natural grouping of the points. The results are shown in Fig. 2, where a periodic signal is apparent. The periodic variation is seen in the contiguous nightly data and all the 1996 data (top panel), in data over the full 1989–96 interval (second panel), and in the phase-mean plots of velocity span and line-depth ratio: the spectral lines of 51 Peg have intrinsic variations with a period of 4.23 d.

The velocity span varies by $\pm 45\text{ m s}^{-1}$, a value comparable to the radial-velocity amplitude found by Mayor and Queloz based on many spectral lines. Because our observations do not tell us if the top or the bottom (or both) of the bisector is moving relative to an absolute frame, the physical phase in Fig. 2 is ambiguous by 180° , and so the near anti-phase with the radial velocities has no immediate significance. One should not even conclude that velocity-span variations translate directly into radial velocity variations. More than likely, there is also a shift of the whole line occurring along with the change in bisector shape, and although this cannot be ascertained directly from my spectroscopic data, such a shift could be the primary effect producing the radial velocity variations.

The physical interpretation of the variation in line-depth ratio is likely to be equally complex, especially if non-radial pulsation is involved. Although line-depth ratios have been used as temperature indicators in previous investigations¹²⁻¹⁴, it would be foolhardy to make the same interpretation here. Oscillatory upheavals in the star's atmosphere could re-shape spectral lines through their Doppler shifts and altered radiation transfer. The lack of brightness variation^{15,16}, provides a strong constraint on

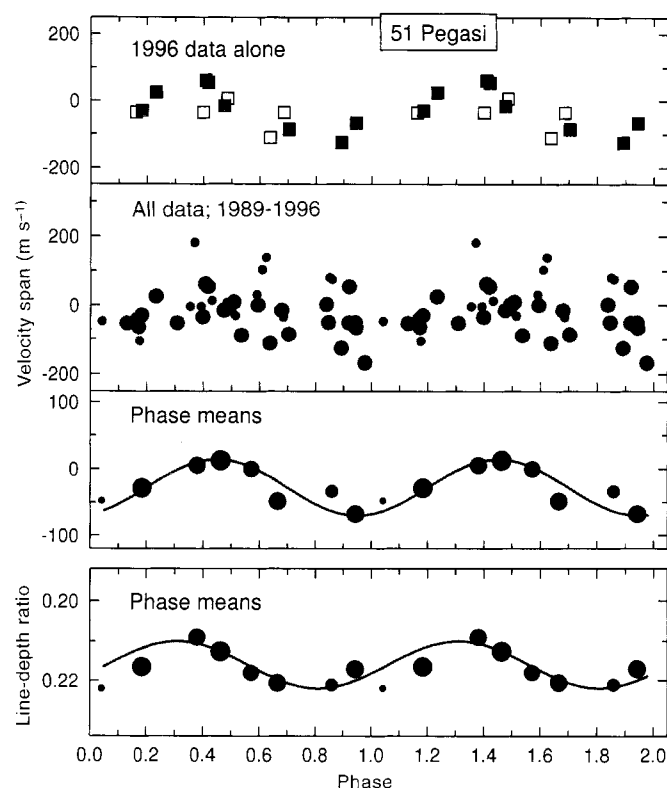


Figure 2 The three upper panels show the velocity span of 6,252.57 Fe I as a function of phase according to the Mayor and Queloz' ephemeris (period of 4.2293 d). The top panel shows just the 1996 data, with contiguous points in time shown by the filled symbols. In the second and third panels, larger symbols are used for higher signal-to-noise ratios of the exposures and their means. In the bottom panel, the ratio of the central depths of the V to the Fe I line is plotted. The cosine curves have the 4.23-d period; the amplitude for the velocity-span curve is 44 m s⁻¹.

modelling these effects.

Regardless of the details of their final interpretation, the 4.23-d spectroscopic variations of 51 Peg are intrinsic to the star; reflex motion of an orbiting planet does not alter the shapes of line profiles.

It is important to realize that high spectral resolving power, >100,000, and high signal-to-noise ratios are needed to see the asymmetries of spectral lines^{8,17}. Radial velocity investigations generally lack this essential ingredient. For example, Mayor and Queloz¹ used a spectral resolution of about half this amount, well below what is needed. At this low a resolving power, the asymmetries of spectral lines virtually disappear. In the one other high-resolution study that has been done, there were simply too few data to be certain of any profile variations¹⁸.

Non-radial pulsations can produce variations in spectral line bisectors that are similar to those seen for 51 Peg¹⁰, although a period of 4.23 d is somewhat larger than one might have guessed based on the solar 5-minute p-mode oscillation. On the other hand, gravity-mode oscillations can have much longer periods¹⁹. Some independent evidence points toward 51 Peg being larger than a normal dwarf star, and this too would give longer periods^{15,16,20,21}. Evidence for low-amplitude long-period pulsations in evolved stars has been seen by several investigators including Walker *et al.*²² The results of non-radial oscillation modelling of these data will be reported elsewhere.

Claims of several other planets outside our Solar System have been made based on this same reflex-motion argument^{23–27}. Periods

of radial-velocity variation range from a few days (for example, τ Bootis, ν Andromedae, ρ^1 Cancri and 51 Peg, through a few weeks and on to a few years for 47 Ursae Majoris and 16 Cygni B. Given the spectroscopic results presented here, non-radial oscillations and possibly rotational modulation are serious contenders for explaining the radial-velocity variations up to a few weeks in length. Rotational modulation becomes an unlikely explanation when the period exceed several tens of days, the normal range for stellar rotation. Non-radial oscillation may not be a viable explanation for periods exceeding a few days, as such long periods are not expected, and because integration of the radial velocity curves imply unreasonably large motion of the stellar surface. However, these kinds of speculations cannot replace high-resolution spectroscopic observations, and they have yet to be done.

Although at this stage, the cause of the spectral line variations in 51 Peg are not fully understood, the chance of their being caused by a planet is vanishingly small. The only hope now for the planet hypothesis, as applied to 51 Peg, is to claim that the spectral variations are somehow driven by the presence of a planet. To make such a claim is actually self-defeating because the intrinsic spectral-line variations will account for most or all of the apparent radial velocity variation upon which the original hypothesis was based. So even if such a claim were to prove true, the planet would be very different from the one originally proposed to explain the radial-velocity variations. On the positive side, it seems likely that the variations shown by 51 Peg will give us an exciting new window into the subtle behaviour of cool stars like our Sun. $\square$

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Uniform elemental analysis of materials by sputtering and photoionization mass spectrometry

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Analysis of the elemental composition of surfaces commonly involves techniques in which atoms or ions are ablated from the material's surface and detected by mass spectrometry. Secondary-ion mass spectrometry^{1,2} is widely used for detection with high sensitivity (down to a few parts per billion) but technical problems prevent it from being truly quantitative. Some of these problems are circumvented by nonresonant laser post-ionization of sputtered atoms followed by time-of-flight mass spectrometry (surface analysis by laser ionization: SALI)³⁻⁹. But when there are large differences in ionization probabilities amongst different elements in the material, the detection sensitivity can be non-uniform and accurate quantification remains out of reach. Here we report that highly uniform, quantitative and sensitive analysis of materials can be achieved using a high-energy (5-keV) ion beam for sputtering coupled with a very-high-intensity laser to induce multiphoton ionization of the sputtered atoms. We show uniform elemental sensitivity for several samples containing elements with very different ionization potentials, suggesting that this approach can now be regarded as quantitative for essentially any material.

In these experiments, steady-state sputtering of bulk materials was used to eliminate any need to consider preferential sputtering, and to ensure sampling over all velocity components of the sputtered material. Examination using a pulsed ion beam showed that steady-state can be reached when the pulse duration exceeds 1 μ s (ref. 10). The photoionization mass spectrometric measurements reflect the number density of atoms and molecules within the laser beam focus because the sputtered species are essentially motionless during the 35-ps laser light 'snapshot'. For quantification, however, the flux is the relevant quantity, which is equal to the number density times the velocity. Conversion from number density to flux is handled assuming all particles leave the surface with the same kinetic energy¹¹. There is some level of uncertainty caused by sputtered dimers or trimers that are detected after photofragmentation by the ionizing beam.

The experiments were performed in an ultra-high vacuum chamber (1×10^{-9} torr) equipped with facilities for standard surface characterization¹². A schematic is shown in Fig. 1. A 5-keV Ar^+ beam was used for both sample cleaning and liberating solid materials into the gas phase (sputtering). Sputtered neutral species were photoionized by high-intensity 532-nm (2.3-eV) light generated from the second harmonic of a 35-ps Nd:YAG laser operated at 10 Hz. The laser beam passes parallel to, and ≤ 1 mm above, the sample surface. Real-time alignment of the focus with the ion optics of the mass spectrometer was performed by leaking 1×10^{-7} torr Ar gas into the chamber and maximizing the Ar^{3+} signal in the mass spectrum while viewing an oscilloscope read-out. Photoions were recorded using a reflecting time-of-flight mass spectrometer. The ionization volume is defined in two dimensions by the laser beam focus and in the third dimension (along the laser beam) by the ion optics. The mass spectrometer's optics are designed to have a sharp cut-off at 4 mm along the laser beam, although in practice, the sputtered plume has effectively smaller dimensions.

The spatial intensity distribution of the laser beam is important to

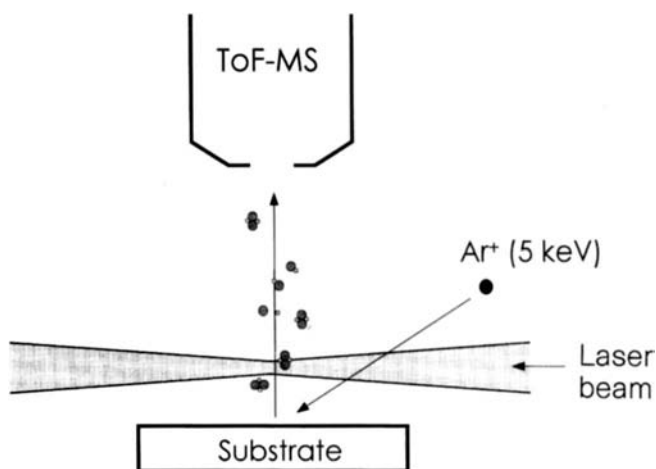


Figure 1 Schematic diagram of the experimental apparatus. The extractor of the time-of-flight mass spectrometer (ToF-MS) was biased at $-1,000$ V and was placed ~ 6 mm above the sample substrate which was electrically grounded. Ar^+ ions impinging at 60° from the surface normal were used for both sample surface cleaning ($0.6 \mu\text{A}$, $400\text{-}\mu\text{m}$ spot diameter rastered over $\sim 2 \times 2 \text{ mm}^2$) and sputtering. The laser beam was focused by a best-form lens with a 10 cm focal length. The full beam waist measured at $1/e^2$ of the peak intensity was $10.6 \mu\text{m}$. With the highest pulse energy of 44 mJ, the laser differential power density reached $2.9 \times 10^{15} \text{ W cm}^{-2}$ at the beam's centre with an average power density over its beam waist of $1.45 \times 10^{15} \text{ W cm}^{-2}$. The averaged power density over the beam waist at the focus was used to characterize the laser power density. Analogue detection of the photoions was used, with photoions impinging on a chevron microchannel plate biased at $-2,400$ V followed by analogue-to-digital conversion by a 100 MHz transient digitizer.

these measurements. To confirm the spatial quality, the laser beam was diagnosed in real-time by directing a small amount of the beam passing through a dielectric mirror to a microscope objective and CCD (charge-coupled device) camera assembly. The intensity distribution of the laser beam at the focal region shows a very near-gaussian, near-diffraction-limited spatial profile¹³. We note that by using wavelengths where at least several photons are required for ionization, the wings of the laser light intensity profile are deemphasized, effectively further sharpening the spatial distribution of the ionization volume.

We have applied this approach to numerous samples (Table 1). The real challenge comes when quantifying elements with very different ionization potentials (IPs) incorporated in a single analysed sample. Some well known examples are SiC, SiO_2 , InTiP and InTiAs^{5,6}. For example, the difference in cross-section (before saturation) for photoionizing C and Si using 532-nm photons, is 10^{30} , whereas the difference amounts to 10^{60} between P and Ti¹⁴. Our results demonstrate that uniform elemental surface analysis can be achieved universally.

Two samples will be explored in detail here: GaAs and SiC single crystals. GaAs is a frequently used semiconducting material; SiC is an insulating material commonly used in microelectronic devices. Figure 2a-c shows typical photoionization mass spectra from a sputtered GaAs sample. The quantification capability of the new data can be evaluated by relative sensitivity factors which are defined, for species S_i in a sample with reference to species S_m , as $\text{RSF}(S_i) = (I_i/[S_i])/(I_m/[S_m])$, where I_i is the summed signal intensity of species S_i in all charged states, and $[S_i]$ is the concentration of species S_i in the sample ($i = 1, 2, \dots, n-1$). A value of unity for RSFs for all species represents completely uniform detection sensitivity, which is an obvious aid in simplifying quantification.

As a laser power density of $4.3 \times 10^{13} \text{ W cm}^{-2}$ (Fig. 2a), the

ionization is not uniform because the signals of Ga^+ are far stronger than the signal of As^+ although the stoichiometry of Ga to As is 1:1. The RSF ratio, derived from peak area integration over all the isotopes, is $\text{Ga}/\text{As} = 8.8/1$. Ga has a first IP of 5.99 eV, requiring three photons for ionization at 532 nm, whereas As has a first IP of 9.81 eV, requiring five photons for ionization. When the laser intensity is increased to $1.32 \times 10^{15} \text{ W cm}^{-2}$, the RSF obtained by peak area integration of singly and multiply ionized isotopes becomes 0.8 for Ga relative to As. The RSF of Ga relative to As as

a function of laser power density is plotted in Fig. 2d. At laser power densities $\geq 3 \times 10^{14} \text{ W cm}^{-2}$, the RSF of Ga approaches unity. At these intensities, multiply ionized atoms have substantial intensities compared with singly ionized atoms, a further indication of saturation of the photoionization of the neutrals.

The experimental mass spectra also confirm that a sharply defined ionization volume has been achieved at the laser focus. When the laser power increases beyond the saturation intensity, the peaks corresponding to singly ionized Ga^+ and As^+ begin to

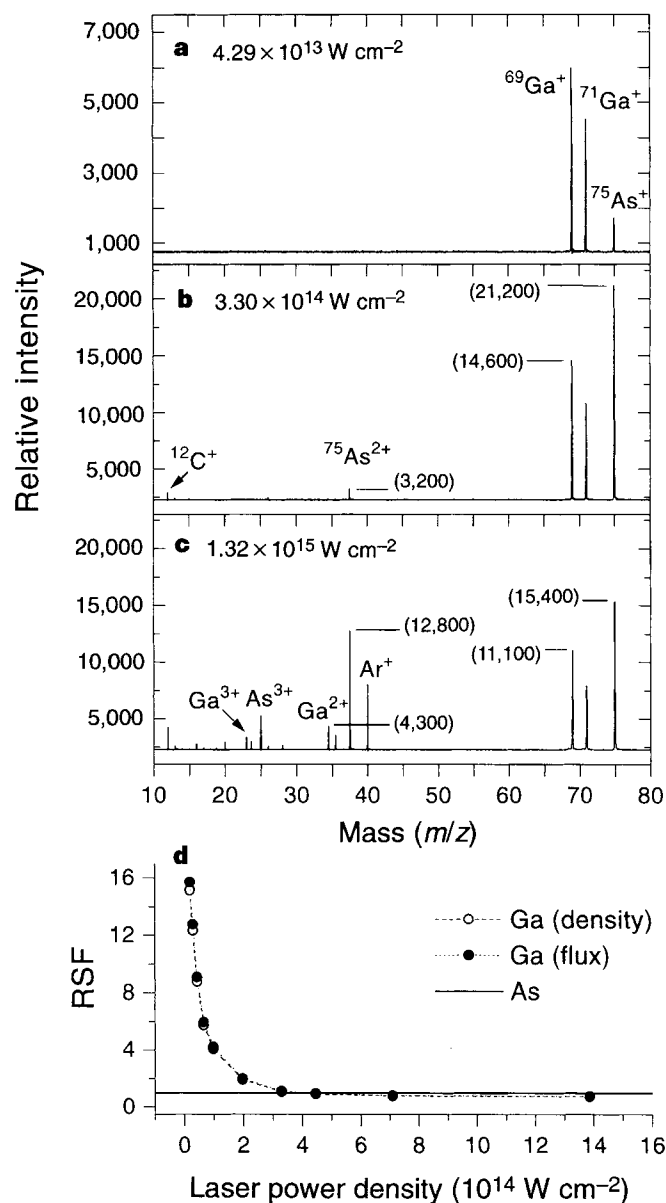


Figure 2 a–c. Nonresonant multiphoton ionization time-of-flight mass spectra from a sputtered GaAs wafer at various laser powers. Each spectrum results from a sum of 200 laser pulses with 30 dB signal attenuation to maintain detection linearity. The intensities are direct read-outs of the 8-bit transient digitizer. The baseline is arbitrary due to the analogue nature of the detection. **d.** Plot of relative sensitivity factors (RSFs) of Ga relative to As as a function of laser power density. RSFs are obtained by peak area integration of all detected photoions. Open circles, direct experimental values; filled circles, values corresponding to a conversion to flux from number density. The correction from a density measurement to a flux measurement is small because the Ga and As are close in mass. The solid straight line represents an RSF of 1 relative to As.

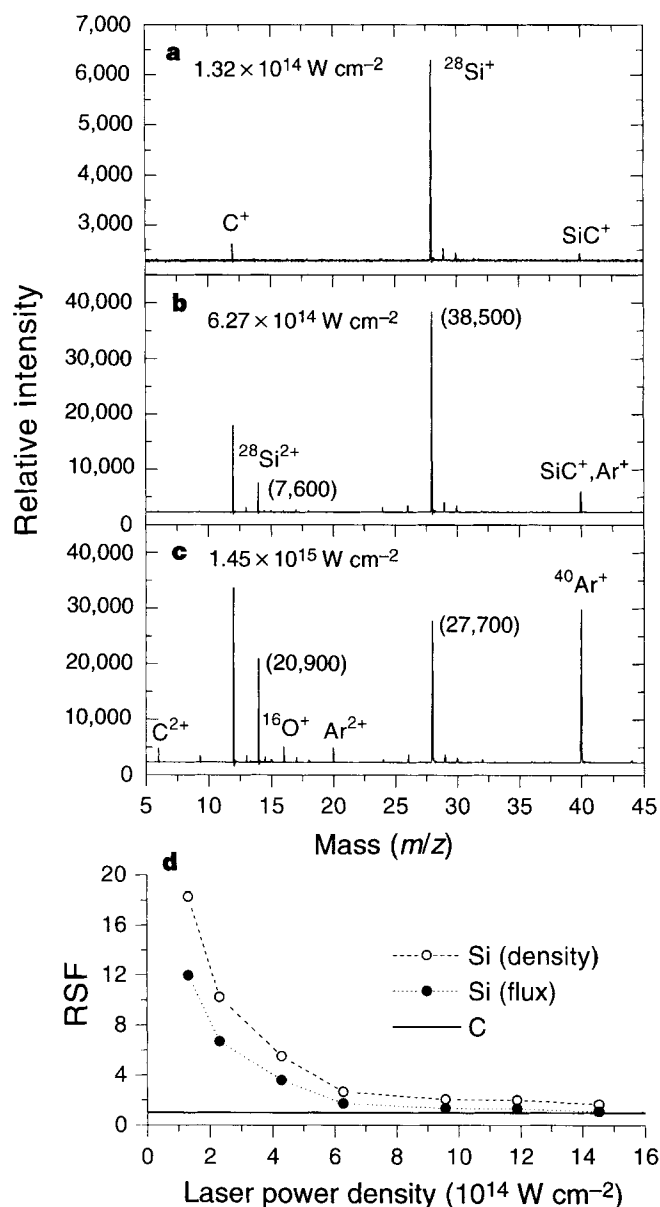


Figure 3 a–c. Nonresonant multiphoton ionization time-of-flight mass spectra of sputtered single-crystal SiC. The spectra result from a sum of 200 laser pulses with 30 dB signal attenuation to maintain detection linearity. **d.** Plot of RSFs of Si relative to C as a function of laser power density. Open circles, direct experimental values; filled circles, values corresponding to a conversion to flux from number density, and the solid straight line represents an RSF of 1 relative to C.

decrease accompanied by the increase of doubly charged Ga^{2+} and As^{2+} . For example, as the laser power density is increased from $3.30 \times 10^{14} \text{ W cm}^{-2}$ to $1.32 \times 10^{15} \text{ W cm}^{-2}$, signal intensities of $^{69}\text{Ga}^+$ and $^{75}\text{As}^+$ decrease from 14,600 and 21,200 counts to 11,100 and 15,400 counts, respectively, while the intensity of $^{69}\text{Ga}^{2+}$ increased from essentially zero to 4,300 counts and the intensity of $^{75}\text{As}^{2+}$ increased from 3,200 to 12,800 counts (Fig 2b, c).

These two observations—that the relative intensity of multiple to single ionization is substantial, and that the signal intensities of the singly charged ion species decrease with increasing laser intensity—contribute strong evidence that the laser beam has created a well defined, fully ionized volume with sharp spatial fall-off in intensity outside this focal volume. Multiply ionized species are believed to be generated by sequential removal of electrons from neutral species^{15,16}.

We observed similar results from a SiC single crystal sputtered with Ar^+ . The IPs of Si and C are 8.151 eV and 11.26 eV, respectively. Figure 3a shows that, at a laser power of $1.32 \times 10^{14} \text{ W cm}^{-2}$, the intensity of C^+ is only about 1/20 of the intensity of Si^+ . When the laser power was increased, the signal intensity of carbon increased substantially (Fig. 3b, c).

The RSFs for Si relative to C are plotted in Fig. 3d as a function of laser power density, with and without the density-to-flux correction. At laser power densities beyond $9.0 \times 10^{14} \text{ W cm}^{-2}$, the ionization seems to be completely saturated. The RSF at $1.45 \times 10^{15} \text{ W cm}^{-2}$ is 1.08 for Si relative to C. As the laser power increases from $6.27 \times 10^{14} \text{ W cm}^{-2}$ to $1.45 \times 10^{15} \text{ W cm}^{-2}$, the signal intensity of $^{28}\text{Si}^+$ decreases from 38,500 to 27,700 counts while the intensity of $^{28}\text{Si}^{2+}$ increases from 7,600 to 20,900 counts (Fig. 3b, c). Again, experimental results demonstrate that not only has ionization of the neutral atoms and molecules been saturated, but that a sharply defined ionization volume has also been created at the laser focus.

High beam quality combined with high laser intensity ($\sim 10^{15} \text{ W cm}^{-2}$) and high-order photoionization are key factors in creating a well defined ionization volume in the ion beam sputtered plumes regardless of ionization potential. Other

measures, such as collecting data at steady-state to eliminate preferential sputtering, sampling to all velocity components, and setting the laser focus region close to the sample to subtend as large a solid angle as possible, also played important roles. Although secondary effects in sampling—such as differences in angular and velocity distributions for the different elements—cannot be totally neglected, the results presented here show the practical ability to drive relative sensitivity factors under very high laser intensity conditions to nearly unity regardless of their ionization potentials. □

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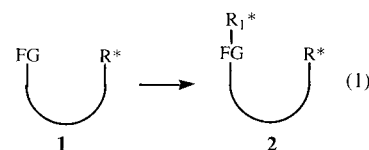
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Induction of molecular asymmetry by a remote chiral group

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Conventional methods of asymmetric synthesis of organic compounds involve the use of either chiral catalysts or stoichiometric amounts of chiral substrates. Invariably, the reacting components must be in close proximity to the entity that introduces chirality^{1,2}. But supramolecular chemistry^{3–5} has shown that stereochemical information can be transmitted through greater distances than are commonly involved in asymmetric syntheses. Here we show that chiral information can be transmitted with remarkable selectivity through as many as nine achiral connecting atoms to give predominantly one diastereomer of a possible four. This contrasts with the general expectation⁶ that a decrease in selectivity would result from an increase in the separation of the chirality-inducing substituent and the reaction site. This ability to convey molecular asymmetry over larger distances should provide access to new chiral molecules that would be difficult to make by conventional methods.



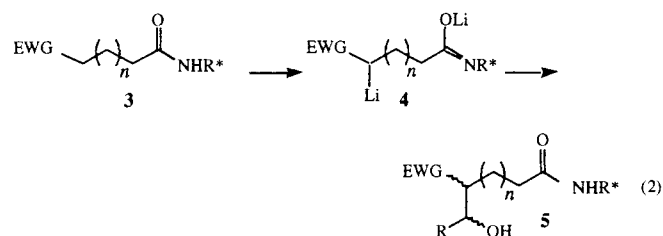
The more general aspects of the long-range asymmetric induction explored here can be represented by equation (1) where R^* is chiral

Table 1 Samples studied

Sample	IP (eV)	RSFs
GaAs	Ga, 6.00 As, 9.81	Ga/As = 0.8/1
SiC	Si, 8.151 C, 11.26	Si/C = 1.08/1
SiO_2	Si, 8.151 O, 13.618	Si/O = 1.23/1
$\text{In}_{0.8}\text{Tl}_{0.2}\text{P}$	In, 5.786 Tl, 6.108 P, 10.486	In/Tl/P = 1.3/1.18/1
$\text{In}_{0.8}\text{Tl}_{0.2}\text{As}$	In, 5.786 Tl, 6.108 As, 9.81	In/Tl/P = 1.3/1.18/1
SRM C1154 stainless steel	Cr, 6.766 Mn, 7.435 Ni, 7.635 Fe, 7.870	Cr/Mn/Ni/Fe = 1.09/0.92/0.84/1
SRM 654A Ti alloy	Ti, 6.82 V, 6.74 Al, 5.986	Ti/V/Al = 1.19/1.13/1
SRM 7075 Al alloy	Cu, 7.726 Cr, 6.766 Mg, 7.646 Zn, 9.394 Al, 5.986	Cu/Cr/Mg/Zn/Al = 1/1.1/0.93/0.9/1

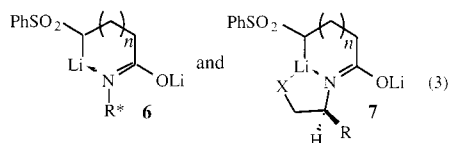
Elemental relative sensitivity factors (RSFs) of various samples studied together with the ionization potentials (IPs) of the relevant elements. GaAs and SiC are single crystals. The alloys are standard reference materials (SRM) in bulk. Data on SiO_2 is obtained from a 400-Å thin film on a Si substrate. Data on $\text{In}_{0.8}\text{Tl}_{0.2}\text{P}$ and $\text{In}_{0.8}\text{Tl}_{0.2}\text{As}$ are obtained from 6,000-Å thin films on InP substrates.

and FG is a functional group that allows a reaction to take place such that **1** is converted into **2** with the creation of new stereochemical information R_1 . Our aim was to identify groups R^* and FG that would enable this kind of interaction. To maximize the interactions between FG and R^* , polar groups could be required, with the necessary proviso that only FG will react. A suitable initial choice for this study is an electron-withdrawing group, and a chiral *s*-amide (equation (2)). Double deprotonation of **3** should give the dianion **4** that can react with an aldehyde to give **5**, where two new stereogenic centres are created. The usual *t*-amide chiral auxiliary cannot be used as it allows amide enolate formation, whereas the *s*-amide is self-protected as **4** (refs 7, 8). We postulated that the dilithio species **4** might exhibit both inter- and intramolecular chelation of the alkylolithium to the imine which would be both solvent- and temperature-dependent⁹.



Initially, we examined two possible modes of intramolecular chelation, the monodentate amide-enolate **6** and the bidentate amide-enolate **7** (equation (3)). We decided that the electron withdrawing group should be $-\text{SO}_2\text{Ph}$ because the β -hydroxyphenylsulphonyl product(s) are usually stable crystalline compounds that should facilitate the stereochemical analysis^{10,11}.

The first set of compounds **8** ($n = 1, 2, 3$ and 4), as 0.1 M solutions in tetrahydrofuran (THF) at -70°C , were individually



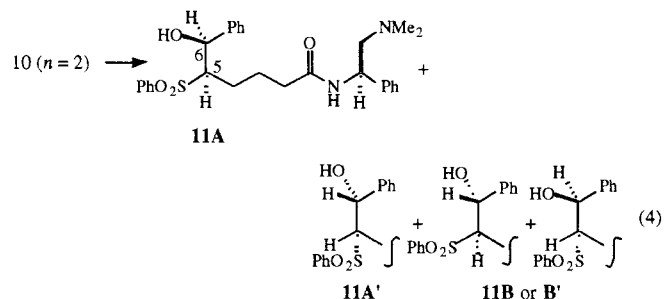
treated with *n*-BuLi (2.2 mol. equiv. in hexanes) followed by benzaldehyde, and the reaction quenched at -70°C (Fig. 1). Analysis of the crude product mixtures by ^1H NMR (300 MHz) revealed two sets of diastereomers of **9** (designated as A/A' and B/B' ; the signals were well separated). The A/A' set shows the respective $\text{PhCH}(\text{OH})$ -proton as two singlets, whereas in the B/B' set it is two doublets ($J = 6\text{--}8\text{ Hz}$)¹⁰. Figure 2a shows a plot of the $A:A'$, $B:B'$ and $A/A':B/B'$ ratios. The most notable feature is the preference (2:1) for the A/A' diastereomers when $n = 2$; when n is 3 and 4, the preference is 1.7:1.

Carrying out the same experiments with hexamethylphosphoramide (HMPA) (2.2 mol. equiv.) added to the dianion followed by benzaldehyde produced virtually no change in any of the diastereomer ratios until $n = 3$ when the $A/A':B/B'$ ratios changed to 4:1. Only a small change in ratios was observed for $n = 4$.

These initial observations, especially the surprising effect of HMPA being confined to the $n = 3$ series, were encouraging and indicative of an aggregate being dissociated to a medium-ring amide anion chelated monomer¹².

The same experiments were conducted with the bidentate ligand series **10**; the results are shown in Fig. 2b. Although the $n = 1$ series did not show any notable differences from **8**, the $n = 2$ series exhibited dramatic diastereomer enhancements. The ratio $A:A'$ (2.2:1) in THF changes to 9:1 when HMPA (2.2 mol. equiv.) was present. Furthermore, the amount of B and B' was reduced such

that the $AA':BB'$ ratio changes from 2.7:1 in THF to 10:1 (HMPA, 2.2 mol. equiv.). This corresponds to a 81.8% yield (by NMR) of diastereomer A. Flash chromatography and crystallization gave **11A** (melting point $115\text{--}117^\circ\text{C}$) whose absolute configuration was determined by X-ray crystallography (Equation (4)). As **11A** has the (5*R*,6*S*) configuration, it follows that **11A'** is (5*S*,6*R*) and **11B/B'** is (5*R*,6*R*/5*S*,6*S*).



Carrying out the above reaction in THF in the presence of either tetramethylethylenediamine (TMEDA) or LiCl did not produce any enhanced ratios¹³. Increasing the amount of HMPA (4.4 mol. equiv.) caused a further improvement in the $A/A':B/B'$ ratio to 19:1 but at the expense of conversion, as the initial products **11** are converted back into the starting materials.

All of these dramatic effects completely disappear in the next homologous series $n = 3$ but are somewhat more modestly restored when $n = 4$; $A:A'$ (4.5:1) (HMPA, 2.2 mol. equiv.) and $A/A':B/B'$ (5.5:1) (HMPA, 2.2 mol. equiv.). This corresponds to a 69.2% yield of **11A** ($n = 4$) (by NMR).

The series derived from *O*-methylisoleucinol **12** ($n = 1, 2, 3$ and 4) gave the results shown in Fig. 2c. When $n = 2$ (HMPA, 2.2 mol. equiv.) the $A/A':B/B'$ ratio becomes 7:1 (compared to 2:1 for $n = 1$), but the $A:A'$ ratio is only 1:1 and therefore the yield of A

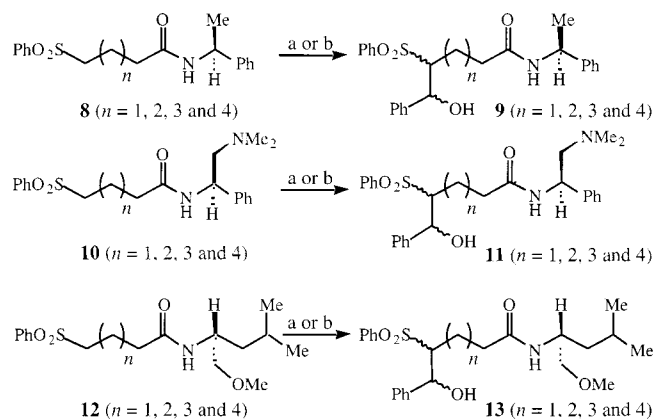


Figure 1 Reaction conditions are as follows: a, THF (0.1 M in substrate)/2.2 mol. equiv. *n*-BuLi/0.5 h/5.0 mol. equiv. PhCHO/ -70°C . b, THF (0.1 M in substrate)/2.2 mol. equiv. *n*-BuLi/0.5 h/2.2 mol. equiv. HMPA/0.5 h/5.0 mol. equiv. PhCHO/ -70°C . Details for **11A**. Under an argon atmosphere **10** (168 mg, 0.43 mmol, $n = 2$) in THF (4.33 ml, 0.1 M) at -70°C was treated with 2.5 M *n*-butyllithium in hexanes (0.38 ml, 0.95 mmol). After 0.5 h, HMPA (0.16 ml, 0.95 mmol) was added to the mixture and the solution stirred at -70°C for 0.5 h. Benzaldehyde (0.22 ml, 2.16 mmol) was added to the mixture at -70°C . After 0.25 h, the mixture was quenched with 2 M HCl (2 ml) at -70°C . The resulting mixture was defrosted, diluted with H_2O (5 ml), and washed with diethyl ether ($2 \times 10\text{ ml}$). The aqueous phase was neutralized with saturated NaHCO_3 and extracted with CH_2Cl_2 ($3 \times 10\text{ ml}$). The combined extracts were dried (Na_2SO_4), and the solvent removed *in vacuo*. This reaction gave **11A**, 81.8% as a single diastereomer by ^1H NMR.

and A' is 43.8%. At $n = 3$ there is a decline in the A/A' : B/B' ratio to 3.7 : 1, which is further reduced when $n = 4$ to 2.3 : 1. In THF alone the A/A' : B/B' ratio for $n = 2, 3$ and 4 stays constant at 2 : 1.

In all the examples above the yields of the major diastereomer are lowest, closest ($n = 1$) to the chiral auxiliary. This is contrary to experience from other asymmetric reactions^{1,2}. The best yields are obtained for the $-NMe_2$ ligand (11A) which has the ability not only to differentiate between the A/A' and B/B' diastereomers, but remarkably between A and A' which are mirror-images at the newly formed stereogenic centres. The high level of enantioselectivity only takes place when $n = 2$ and 4.

The alternating nature of the selectivity implies that the bidentate model 7 (equation (3)), is favoured over intermolecular chelation when it is a 10- or 12-membered ring ($n = 2$ and 4) versus 9- and 11 ($n = 1$ and 3). (Both $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for the formation of medium-ring lactones exhibit alternating behaviour)¹⁴. This suggests that it should be possible, through the design of appropriate ligand sizes,

to select specific chain lengths for long-range asymmetric induction. Although the above reactions all involve α -sulphonyl carbanions, it is rather unlikely that the results reported here are confined to this functionality. Furthermore, it should be feasible to study reactions that operate under equilibrium conditions that might favour a particular stereochemical outcome through hydrogen bonding. $\square$

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Eccentricity forcing of Pliocene–Early Pleistocene climate revealed in a marine oxygen-isotope record

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Milankovitch theory—that climate is controlled by variations in the Earth's orbital parameters—has gained wide acceptance for its ability to account for two climate cycles: a 23-kyr cycle that is phase-locked to the precession-driven insolation cycle, and a 41-kyr cycle that is phase-locked to the obliquity-driven insolation cycle^{1–6}. But, explaining the observed ~100-kyr climate cycle in terms of Milankovitch theory—especially for the Late Pleistocene ice-age cycle—remains controversial in spite of a strong correlation with the ~100-kyr cycle in the Earth's orbital eccentricity⁵. One problem is that eccentricity affects insolation mainly by modulating the precession cycle; its direct contribution to radiation change is too small (<0.1%) to effect the observed climate change directly^{5,7}. Another is the absence of a Late Pleistocene ice-volume cycle in oxygen-isotope records to match the ~404-kyr component of the eccentricity cycle^{5,8}. Here we examine an oxygen-isotope record spanning the interval 1.2 to 5.2 million years ago, before the Late Pleistocene ice-age regime. We find 404-kyr and ~100-kyr climate cycles which are coherent with eccentricity and which have amplitudes that are similar to the coexisting 23-kyr cycle. Analysis of these low-frequency cycles suggests that they originate through an asymmetrical response mechanism that preferentially introduces variance into the climate system from the warmer portions of the eccentricity-modulated precession cycle. Our data thus support eccentricity's role in the origin of low-frequency oxygen-isotope cycles before the Late Pleistocene ice age.

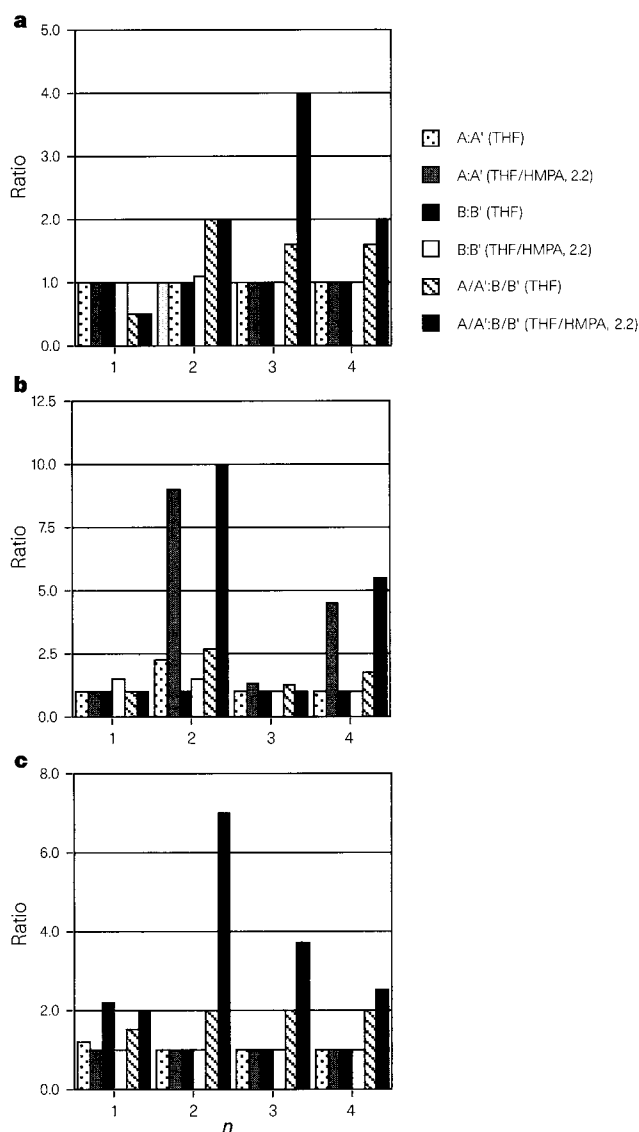


Figure 2 The ratios of diastereomers formed as a function of n . **a**, For compound **9**; **b**, for **11**; and **c**, for **13**. Structures of these compounds are shown in Fig. 1. All of the products are stable to the reaction conditions and do not equilibrate, and therefore represent the kinetic products. But when >3 mol. equiv. of HMPA is added the starting materials reappear, which indicates equilibration.

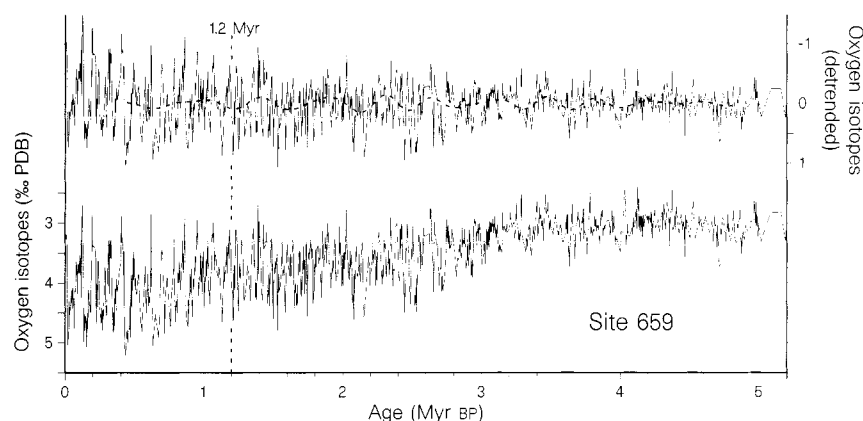


Figure 1 Benthic (*Cibicoides wuellerstorfi*) $\delta^{18}\text{O}$ record from Ocean Drilling Program Site 659 (18°05' N, 21°02' W, 3,070 m water depth). Age control is based on oxygen-isotope chronostratigraphy (0–2.85 Myr) and tuning the lithogenic component to 65°N July insolation (2.85–5.2 Myr), as described by Tiedemann *et al.*¹¹. Lower curve, $\delta^{18}\text{O}$. Upper curve, detrended $\delta^{18}\text{O}$ used in spectral and cross-spectral analyses; dashed line is the 404-kyr bandpass filter. Detrending was done with a locally weighted fit (least-squared error). The ice-age regime, characterized by large-amplitude ~100-kyr cycles, spans 0–1.2 Myr. Before 1.2 Myr, $\delta^{18}\text{O}$ fluctuates with a dominant cyclicity of 41 kyr.

Models developed to investigate the ~100-kyr ice-age cycles of the past 1.2 Myr range from those that invoke orbital forcing to those that rely on free oscillations internal to the climate system (see ref. 5 for a review). Models with free, self-sustaining oscillations explain the ~100-kyr cycle as a response to internal climate-system interactions (for example, between ice sheets, ocean circulation and atmospheric CO_2) whose phase is arbitrary with respect to eccentricity because orbital forcing plays no role. Such models can explain the apparent absence of the 404-kyr period but have difficulty accounting for the significant coherence and phase lock which clearly exist between the ~100-kyr periods of eccentricity and oxygen isotopes ($\delta^{18}\text{O}$) (ref. 5). Models that invoke orbital forcing explain the cycle as a nonlinear interaction between orbital insolation and internal dynamics of the atmosphere, oceans, ice sheets and lithosphere. Nonlinearities inherent in these models result in predicted climate variations that span the full range of orbital periods including the 404-kyr period (as well as harmonics of the primary eccentricity periods). Inclusion of the 404-kyr period has been generally considered a drawback of this class of models because it is not observed in the $\delta^{18}\text{O}$ record of the past 1.2 Myr.

With the recovery of long, undisturbed deep-sea sediment cores, we can now examine the $\delta^{18}\text{O}$ record before the onset of the Late Pleistocene ice ages, during a regime when ~100-kyr variability in $\delta^{18}\text{O}$ existed but was not greatly amplified as in the Late Pleistocene^{9–11}. Here we note that, before 2.7 Myr, the extent to which benthic $\delta^{18}\text{O}$ reflects changes in deep-ocean temperature, as opposed to changes in global ice volume, is not clear. Records of ice-rafted detritus in deep sea sediments indicate the presence of continental ice-masses, large enough to reach sea level, adjacent to the north Atlantic¹² and north Pacific¹³ oceans as far back as 5.5 Myr. Although both records indicate that the major intensification of Northern Hemisphere glaciation began ~2.7 Myr ago, they also show that Northern Hemisphere ice volume has contributed to the $\delta^{18}\text{O}$ signal since ~5.5 Myr. In any case, as both ice-volume and deep-water temperature signals emanate from high-latitude sources during the Plio–Pleistocene, the benthic $\delta^{18}\text{O}$ record is a good proxy for changes in high-latitude climate.

The site 659 $\delta^{18}\text{O}$ record (Fig. 1) displays the well documented long-term increase reflecting the combined effects of deep-ocean cooling and increased continental ice volume (see, for example, refs 9, 11, 14). Superimposed on the long-term trend are $\delta^{18}\text{O}$ oscillations with periods near 41 kyr and 23 kyr, associated with orbital obliquity and precession (see, for example, ref. 4). Here, we concentrate specifically on variance near the 100- and 404-kyr periods, using spectral analysis to evaluate its relationship to orbital eccentricity and insolation. The length of the Site 659 record is a great advantage as it provides ten 404-kyr cycles to establish the statistical significance of variability in that spectral band.

Because spectral analysis over a given time interval assumes

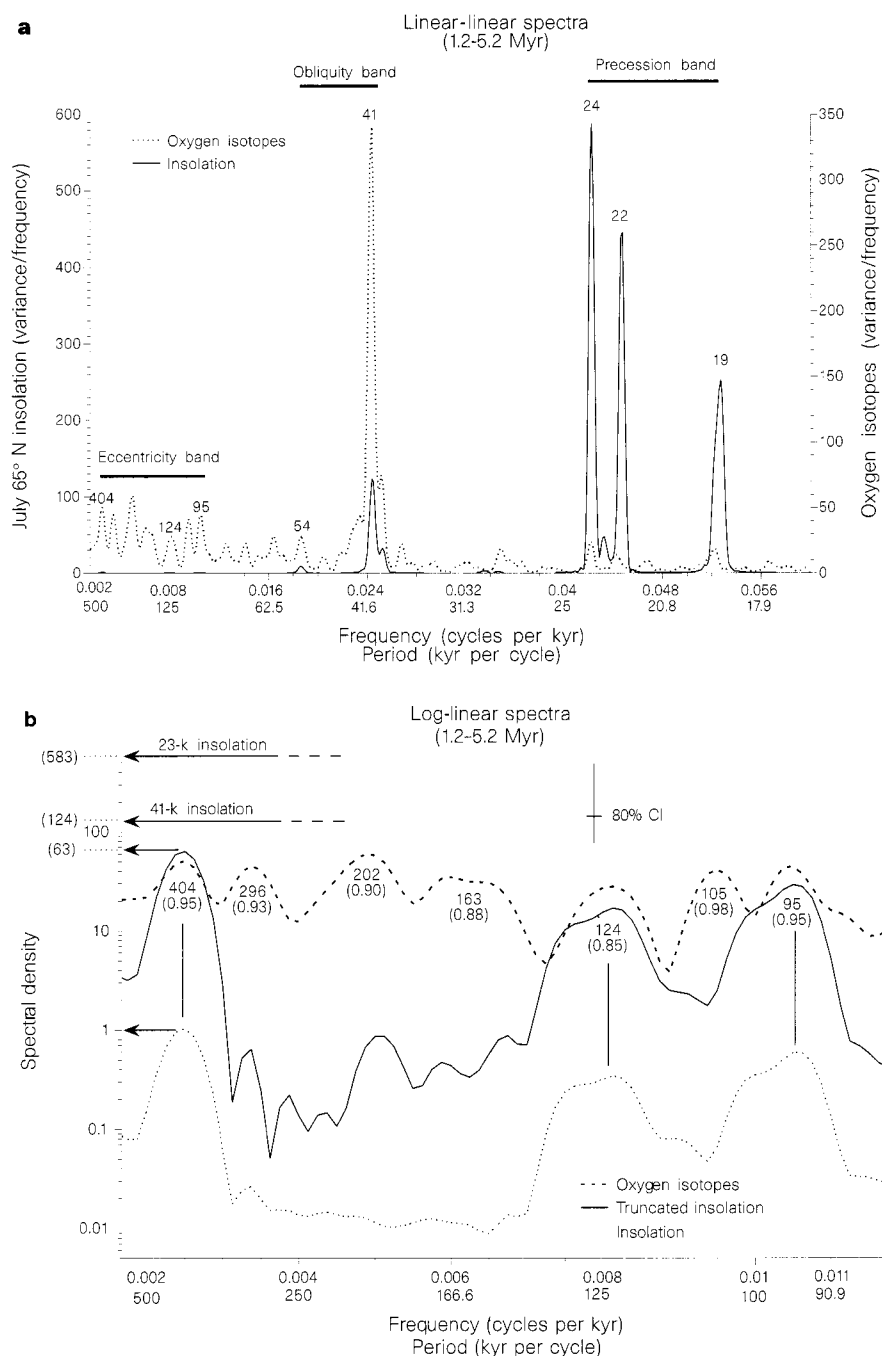
stationarity (constant mean and variance), we first detrend the Site 659 $\delta^{18}\text{O}$ record (Fig. 1). The detrended time-series and its associated bandpass filter reveals low-frequency (~400-kyr) cyclicity before 1.2 Myr. This cyclicity, also present in the non-detrended data, is largely defined by heavy (more positive) isotopic values indicating increased ice volume and/or colder deep-ocean temperatures. The ~400-kyr cyclicity appears strongest between 4 and 1.2 Myr with the exception of a non-stationary interval between 3.3 and 2.6 Myr where the $\delta^{18}\text{O}$ amplitude markedly increases, a non-stationary aspect of the data not removed by the detrend. Although this cyclicity can be seen by eye, spectral analysis provides a more rigorous evaluation of the relative concentrations of variance across all frequency bands.

Linear–linear spectra (Fig. 2a) show the relative concentrations of variance in insolation (July 65°N) and $\delta^{18}\text{O}$ over the interval 1.2–5.2 Myr. Insolation variance is concentrated in the precession band (24, 22 and 19 kyr) and obliquity band (54 and 41 kyr); the insolation variance which does exist in the eccentricity band (404, 124 and 95 kyr) is too small to be seen in this plot. The $\delta^{18}\text{O}$ spectrum shows concentrations of variance not only in the obliquity and precession bands, as has been well documented, but in the eccentricity part of the spectrum as well.

A log-linear spectra of the eccentricity-band region (Fig. 2b) brings out the small amount of insolation variance directly associated with eccentricity. Significantly, the $\delta^{18}\text{O}$ spectrum has peaks at 404, 124 and 95 kyr, matching the three strongest peaks in orbital eccentricity³ in terms of period and relative spectral density; the 404-kyr peak is the strongest and 124-kyr peak the weakest of the three. Probabilities that line frequencies exist within the spectral peaks¹⁵ range from 0.85 for the 124-kyr peak to 0.95 for the 404- and 95-kyr peaks. Combined, the 124- and 95-kyr peaks represent the so-called '100-kyr' peak cited in literature. Resolved spectral peaks also exist at periods for which there is no corresponding concentration of insolation variance (296, 202, 163 and 105 kyr). Probabilities for these peak are 0.93, 0.90, 0.88 and 0.98, respectively. We observe similar 'non-primary' spectral peaks in $\delta^{18}\text{O}$ records from North Atlantic Site 607 and equatorial Pacific Site 846^{9,16}. Although the significance of these peaks is not clear, we speculate that the 202-kyr peak may represent the second harmonic of the 404-kyr cycle and that the 105-kyr peak may represent a beat of the 1/296-kyr and 1/163-kyr frequencies. These await further analysis. Here we concentrate on variance associated with the primary eccentricity periods.

Given that eccentricity (e) modulates the amplitude of precession ($e \sin \omega$, where ω is the longitude of perihelion), the full suite of eccentricity peaks in the $\delta^{18}\text{O}$ spectrum suggests that the pre-1.2 Myr climate system transfers variance from the upper envelope of precession-dominated insolation into the 404-, 124- and 95-kyr eccentricity bands. This suggestion is supported by the observation that the amplitudes of the 404-, 124- and 95-kyr $\delta^{18}\text{O}$ cycles are

Figure 2 Spectral-density plots comparing insolation for July 65° N and Site 659 $\delta^{18}\text{O}$ over the interval 1.2–5.2 Myr. The use of July 65° N stems from the basic mechanism proposed by Milankovitch: that periods of low summer insolation in high latitudes precludes melting, resulting in a positive ice-budget, glacial advance, and positive albedo feedback. Spectra were generated using the Blackman–Tukey method²⁰ with a bandwidth of 0.00066 kyr^{-1} . **a**, Linear-linear plot: insolation variance is dominated by precession with lesser amounts of obliquity, $\delta^{18}\text{O}$ variance is concentrated in the obliquity and precession bands as well as in the low-frequency (eccentricity) region of the spectrum. **b**, Low-frequency (eccentricity band) portion of the spectra **a** in log-linear form and including the spectrum of truncated insolation (July 65° N; all values below the mean set to zero). Insolation variance within the eccentricity band (404-, 124- and 95-kyr periods) is strongly amplified in the truncated record. Spectral peaks are labelled. Numbers in parentheses are *F*-statistic probabilities that line frequencies exist within the $\delta^{18}\text{O}$ spectral peaks¹⁵. As evidence that the spectral power in the eccentricity band is independent of the astronomical age model (Fig. 1 legend), we note that the location and coherence of the $\delta^{18}\text{O}$ spectral peaks (Figs 2b and 3) remain stable as the number of control points in the astronomical age-model (162) is systematically reduced by 50%, 75%, 87% and 93%. Thus, the same results are achieved with as few as 11 control points distributed throughout the 4 Myr record.



approximately equal to the amplitude of the 23-kyr $\delta^{18}\text{O}$ cycle, each accounting for $\sim 0.1\%$ of the total 0.6% amplitude. The remaining 0.2% is accounted for by the amplitude of the 41-kyr cycle.

Mathematically, transfer of spectral variance from the precession band to the modulating eccentricity band can be accomplished by simple truncation or clipping. For example, truncating July 65° N insolation yields concentrations of variance in the eccentricity band which are significantly increased relative to variance in the obliquity and precession bands (Fig. 2b). Cross-spectral comparison of truncated insolation and $\delta^{18}\text{O}$ indicates significant coherence at the 404-, 124- and 95-kyr periods; coherence in the 404-kyr band surpasses the 95% level (Fig. 3). This suggests an asymmetric (truncated) response to insolation whereby the $\delta^{18}\text{O}$ response is less sensitive to colder portions of the insolation cycle than to warmer portions, a type of mechanism prevalent in many orbital forcing models. Further support for a truncation mechanism stems

from the potential presence of line frequencies¹⁵ in the $\delta^{18}\text{O}$ multitaper spectrum at 10.3 kyr (probability, 0.98) and 11.6 kyr (probability, 0.99) which is also consistent with an asymmetric response to insolation forcing.

The energy balance model of Short *et al.*¹⁷ provides an excellent example of a truncation process within the Earth's climate system. Model results indicate a substantial transfer of variance from the precession to the eccentricity bands within the maximum temperature response of the tropics. In this case the transfer is due to the interaction of perihelion with the overhead passage of the Sun at either equinox. This serves to truncate the effects of the lower (cooler) portions of the tropical insolation cycle resulting in concentrations of power in the temperature spectrum at 400- and ~ 100 -kyr periods. These results are consistent with spectra of tropical aridity which show strong concentrations of variance in these bands (see, for example, ref. 11). Various mechanisms exist by

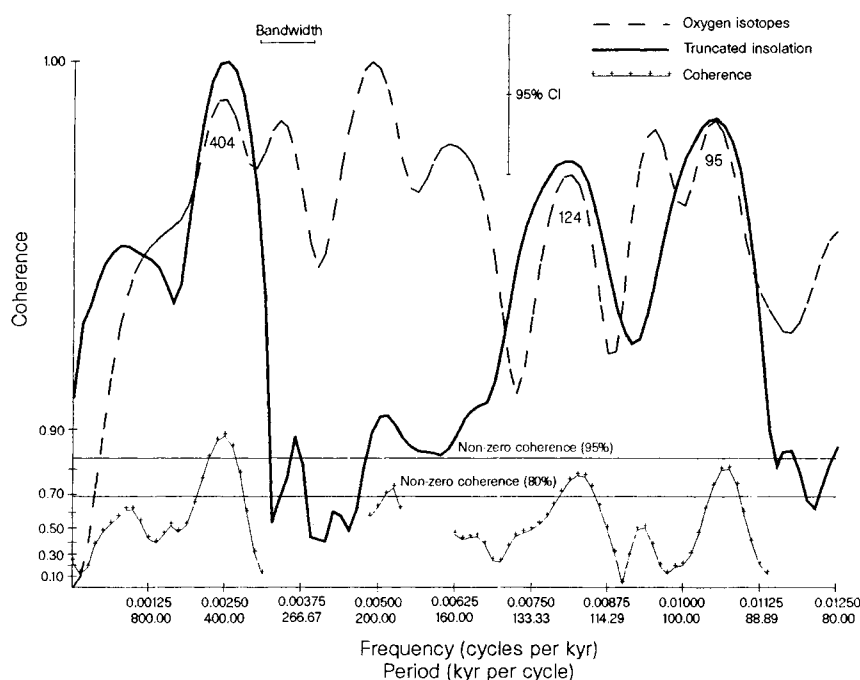


Figure 3 Cross-spectral comparison of Site 659 $\delta^{18}\text{O}$ and truncated insolation (July 65°N) for 1.2–5.2 Myr. Cross-spectrum generated using Blackman–Tukey method²⁰ with a bandwidth of 0.00089 kyr^{-1} . Spectral densities (variance/frequency) are normalized and plotted on log scales. The coherence spectrum (y-axis; solid line with crosses) is plotted on a hyperbolic arctangent scale. The solid horizontal lines denote test-statistics for non-zero coherence.

which the tropical temperature response could be exported to high latitudes thus influencing ice volume^{17–19}. Ice–albedo feedback may also be involved in truncating the effects of high-latitude insolation forcing, providing a high-latitude means of transferring variance via truncation.

The $\delta^{18}\text{O}$ spectrum of the ice-age regime (0–1.2 Myr) indicates little, if any, measurable 404-kyr cycle and a highly amplified ~100-kyr cycle. In contrast, the $\delta^{18}\text{O}$ spectrum from 1.2 to 5.2 Myr indicates 404-, 124- and 95-kyr cycles, closely matching the three main eccentricity cycles in terms of period and relative spectral density. This suggests that variance in the insolation forcing (concentrated largely in the 23-kyr band) is shifted into the modulating eccentricity band, possibly by a truncation mechanism associated with interactions among internal climate components and external insolation forcing. Thus the orbital class of models, which invoke a simple nonlinear response to insolation forcing, seem appropriate for explaining the pre-ice-age climate spectrum as recorded by $\delta^{18}\text{O}$. Model output including the 404-kyr cycle, considered a drawback as applied to the ice-age regime, appears consistent with the climate spectrum of the previous 4 million years. □

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Vegetation-induced warming of high-latitude regions during the Late Cretaceous period

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Modelling studies of pre-Quaternary (>2 million years ago) climate implicate atmospheric carbon dioxide concentrations¹, land elevation² and land–sea distribution^{3–5} as important factors influencing global climate change over geological timescales. But during times of global warmth, such as the Cretaceous period and Eocene epoch, there are large discrepancies between model simulations of high-latitude and continental-interior temperatures and those indicated by palaeotemperature records^{6,7}. Here we use a global climate model for the latest Cretaceous (66 million years ago) to examine the role played by high- and middle-latitude forests in surface temperature regulation. In our simulations, this forest vegetation warms the global climate by 2.2°C . The low-albedo deciduous forests cause high-latitude land areas to warm, which then transfer more heat to adjacent oceans, thus delaying sea-ice formation and increasing winter temperatures over coastal land. Overall, the inclusion of some of the physical and physiological climate feedback effects of high-latitude forest vegetation in our simulations reduces the existing discrepancies between observed and modelled climates of the latest Cretaceous, suggesting that these forests may have made an important contribution to climate regulation during periods of global warmth.

Simulations were conducted using a coupled ocean–atmosphere–sea-ice general circulation model (National Center for Atmospheric Research GENESIS Global Climate Model, Version 1.02)⁸. GENESIS includes an atmospheric model, a mixed-layer ocean model with prescribed ocean heat transport, multi-layer models of sea ice, snow

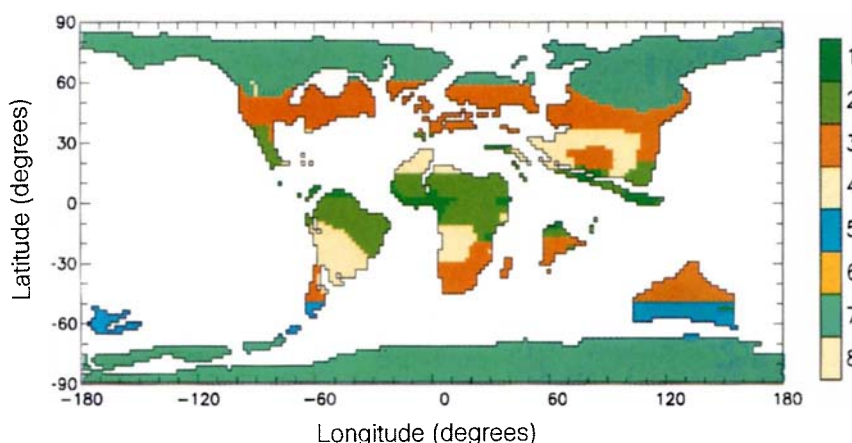


Figure 1 Estimate of Maastrichtian vegetation (used in the BESTGUESS simulation) derived from >300 records of lithological and palaeontological indicators. The vegetation types 1–8 (see colour scale on right-hand side of figure) and their correspondence with the modified Köppen scheme are as follows: 1, tropical rain forest = Af; 2, tropical semi-deciduous forest = Am, Aw; 3, broad-leaved evergreen woodland = Cfa; 4, desert and semi-desert = BW, BS; 5, temperate evergreen conifer and angiosperm forests = Cfb-c; 6, savanna (not used in simulations) = Aw; 7, polar deciduous forest = Dfa-d; 8, bare soil (used to code cold deserts) = BS, BW. Biophysical parameters for vegetation types are derived from ref. 10 and are described in Supplementary Information.

and soil, and a land-surface transfer model that simulates the physical effects of vegetation⁹. Vegetation is represented by an upper layer of trees or shrubs and a lower layer of grass or bare soil. Prescribed variables include vegetation type, leaf area index, fractional coverage, canopy height, solar transmittance/reflectance and stomatal resistance¹⁰. The vegetation influences the exchanges of radiative and turbulent fluxes between the surface and the atmosphere; it can respond to climate physiologically but cannot respond by changing fractional cover, leaf area or life-form abundance.

The latest Cretaceous atmospheric partial pressure of CO₂ in simulations is twice pre-industrial levels (580 p.p.m.), a conservative figure that is slightly higher than the 'best estimate' of geochemical models¹¹ and significantly lower than estimates of isotopic proxies¹². This conservative value avoids strong greenhouse warming of high latitudes allowing better evaluation of the climatic effects of forest vegetation. The palaeogeography is drawn for the latest Maastrichtian stage using an update of earlier plate-tectonic reconstructions¹³ and regional palaeogeographies. Palaeoelevations are set to 250 m for lowlands, and 1,000 and 2,000 m for uplands, a compromise between two palaeogeographical formulas^{14,15}. Solar insolation conforms to present-day solar orbital configuration with a solar constant 0.664% less than at present¹⁶.

The model simulations are used to determine the vegetation's contribution to climate. One simulation, BARESOIL, provides baseline statistics for an unvegetated Earth; it is the boundary condition used in most earlier simulations of Late Cretaceous climate. Soil texture and colour are set to intermediate values with an average snow-free land albedo of 15%. The second simulation, BESTGUESS, uses a 'best estimate' of global vegetation during the Maastrichtian (see Fig. 1 and Supplementary Information) to determine the contribution of areally widespread high-latitude forest to Late Cretaceous global warming^{17–19}. Fossil vegetation is categorized by biome and assigned structural and physiological parameters of the most similar extant biome(s)¹⁰. The understorey layer for each vegetation type is coded as bare soil because the land-surface transfer model employed only recognizes grass or bare soil for understorey. Fossil grasses do not appear until the Tertiary period^{20,21}.

Vegetation increases global mean temperature by 2.2 °C relative to bare soil (Table 1). The tropics warm an average of 1.5 °C, while high latitudes (60°–90°) warm 4.1 °C in the Northern Hemisphere and

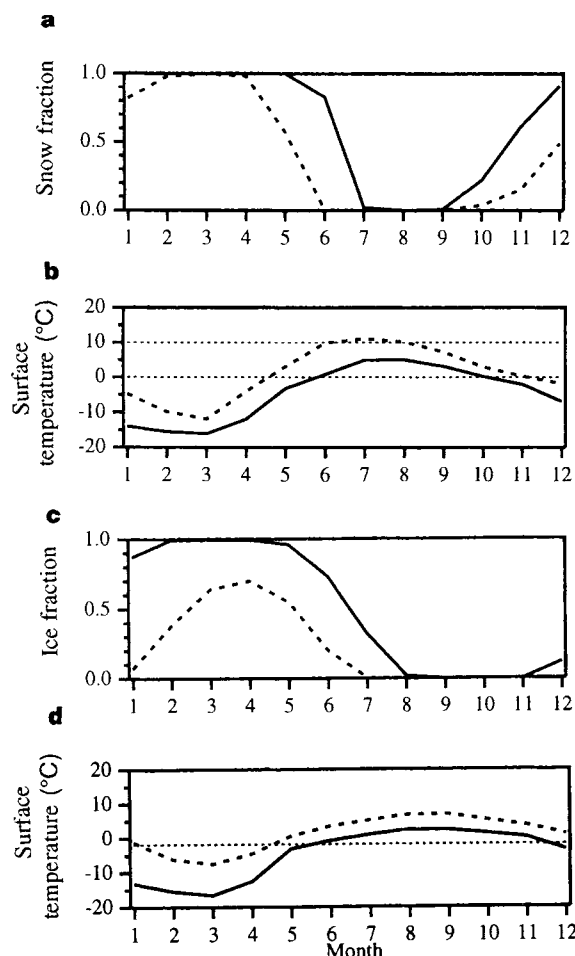


Figure 2 BESTGUESS (dashed lines) and BARESOIL (solid lines) simulations, annual cycles (month 1 is January). **a**, Fractional snow coverage at 67° N, 151° W (Alaska). **b**, Surface temperature at 67° N, 151° W (Alaska). **c**, Fractional sea-ice coverage at 67° N, 164° W (North Pacific). **d**, Surface temperature at 67° N, 164° W (North Pacific). Dotted lines are 0 °C (freezing) and 10 °C (tundra-forest boundary) isotherms for Alaska plot and -1.8 °C (sea-ice formation) isotherm for North Pacific plot.

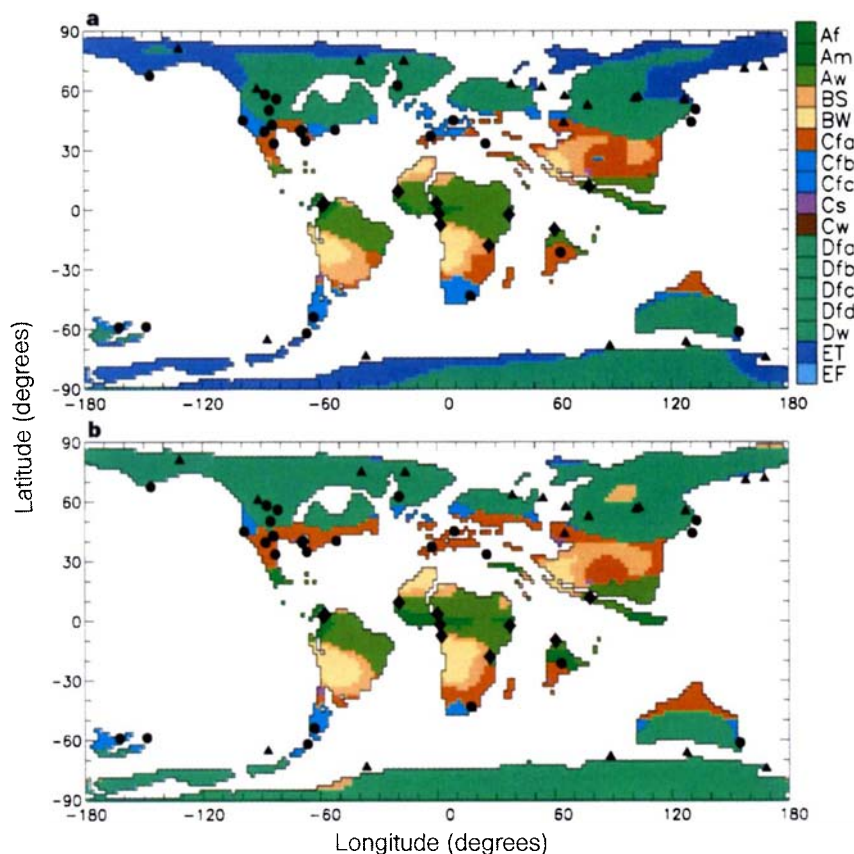


Figure 3 Model climates and the distribution of forest/woodland vegetation (**a**, BARESOIL; **b**, BESTGUESS). Climates (see colour scale at right-hand side of **a**) correspond to our modified Köppen scheme^{22,23}. Cretaceous equatorial forests are inferred largely from coals and tree ferns. Other forests are inferred from leaves with forest-type physiognomy, fossil tree stumps >0.5 m in diameter, or at least 2–3 pollen types assignable to extant tree genera. At middle latitudes, warm-temperate (C) forests are inferred from plant megafossils that indicate cold-month mean temperatures >1°C (for example, palms, gingers, large-leaved evergreen dicots, woods without annual rings) or mean annual temperatures >20°C. Detailed vegetation data are given in Supplementary Information. Diamonds, tropical forest; circles, warm-temperate forest; triangles, cool-temperate forest.

3.4°C in the Southern Hemisphere. High-latitude land surfaces in the Northern Hemisphere warm year-round in BESTGUESS (annual mean warming, 4.2°C) with above-freezing temperatures present for two additional months. High-latitude oceans warm by transfer of energy from warmer continents, 4.0°C in the Northern Hemisphere and 3.5°C in the Southern Hemisphere (Table 1). Vegetation reduces areal extent and duration of sea ice.

Forest vegetation induces feedbacks between surface energy exchanges, surface temperatures, and snow and ice cover (see Fig. 2 and Supplementary Information). For southern coastal Alaska, forest vegetation reduces surface albedo, especially from January until the end of June. In late winter–early spring, trees partially mask snow and absorb more incoming radiation. In late spring–early summer, earlier spring melting of snowcover lowers surface albedo and increases absorbed solar radiation (78% increase in April–June for BESTGUESS), an increase only partially offset by increased energy fluxes from the forested land surface (Fig. 2a). Surface temperatures in BESTGUESS rise above freezing in late April and remain above freezing until late October (Fig. 2b); those in BARESOIL remain below freezing until early June and stay above freezing only until late September.

High-latitude land surfaces warm adjacent oceans, which induces feedbacks between sea surface temperature, ice cover, surface albedo and surface heat exchanges. In BESTGUESS the ocean just west of Alaska never completely freezes; sea-ice coverage exceeds 50% only from mid-February to early May (Fig. 2c). A net warming (Fig. 2d) from April to June results from decreased ocean surface albedo (from 0.61 to 0.48) and increased absorbed solar radiation (from 100 to 150 W m⁻²). Warmer ocean waters in late summer to early winter inhibit sea-ice formation during late winter and early spring (Fig. 2c).

The Köppen system²² correlates present-day vegetation with climate using monthly mean temperature and precipitation. The

GENESIS model reproduces the present-day distribution of Köppen climate types, especially boundaries between warm temperature (C), cool temperate (D) and tundra (E) climates. Regional features, such as rain-shadow deserts and the Indian summer monsoon, are inadequately simulated owing to model-smoothing of mountains. This deficiency is not critical for evaluation of mid- and high-latitude climates. Comparison of model climate with palaeovegetation is based on a modified Köppen system where a cold-month mean of 1°C separates warm-temperature (C) and cool-temperate (D) climates; a warm-month mean of 20°C separates warm-temperate (Cfa) and marine (Cfb, Cfc) climates²³; and the sub-humid/humid boundary is modified to compensate for overestimation of precipitation by the model.

In BARESOIL (Fig. 3a), cool-temperate climates with severe winters and broad-leaved deciduous or boreal conifer forests dominate Australia, Europe and the northeastern United States and expand into Antarctica. Tundra is predicted in Asia and on the fringes of North America and Antarctica. In BESTGUESS (Fig. 3b), warm-temperate climates migrate poleward and replace cool-temperate climates in North America and Australia. Cool-temperate climates replace tundra climates in Alaska, northern Canada, and Asia. Marine climates replace tundra climates on the Antarctic peninsula.

Excellent correspondence exists between input ‘best-estimate’ vegetation and tropical and dry climates predicted by BESTGUESS. Atmospheric dynamics and surface exchanges controlling tropical and subtropical climates are well represented in the model. Cool-temperate climates are also correctly simulated by BESTGUESS. Simulated climates between 35° and 55° latitude in BESTGUESS, however, are too cool to support the warm-temperate broad-leaved evergreen forests prescribed in the model run. This discrepancy between model output and prescribed vegetation is documented by over 20 fossil sites in North America, Asia and Australia.

Table 1 Simulated mean annual climate statistics for years 14–20

Experiment	BARESOIL	BESTGUESS
Surface temperature (°C)		
Global	15.8	18.0
60° N–90° N	–5.5	–1.4
15° N–15° S	26.1	27.6
60° S–90° S	–0.8	2.6
Land 60° N–90° N		
Surface temperature (°C)	–7.5	–3.3
Fractional snowcover	0.62	0.52
Land 60° S–90° S		
Surface temperature (°C)	–5.8	–2.5
Fractional snowcover	0.63	0.52
Ocean 60° N–90° N		
Surface temperature (°C)	–3.3	0.7
Ice fraction	0.38	0.23
Ocean 60° S–90° S		
Surface temperature (°C)	1.8	5.3
Ice fraction	0.17	0.06
Ocean 15° N–15° S		
Surface temperature (°C)	25.8	26.9

Simulations reached equilibrium by year 14. Temperature values are for 2 m above the land or ocean surface.

Inclusion of vegetation in simulations markedly improves correspondence between model climate and palaeovegetation for regions where climate boundaries are delimited by summer temperature. BARESOIL (Fig. 3a) incorrectly predicts tundra for high northern latitudes and the Antarctic coast, regions where fossil assemblages indicate forests (and hence warm-month means $>10^{\circ}\text{C}$). BESTGUESS (Fig. 3b), in contrast, correctly predicts the distribution of forest vegetation at high latitudes. No evidence exists for low-elevation tundra vegetation.

Winter temperatures in BESTGUESS are warmer than those in BARESOIL but still too cold. Megafossils of plants sensitive to freezing, including palms, gingers and broad-leaved evergreen trees^{7,24}, occur poleward of the 1°C cold-month mean for much of North America as simulated in BESTGUESS. Palaeobotanical estimates of mean annual temperature for North America^{19,25–27} exceed model estimates at high latitudes. This conflict may result from: (1) unrealistically low levels of prescribed CO_2 partial pressure, (2) insufficient prescribed ocean heat transport, or (3) failure of general circulation models to incorporate critical processes that affect atmospheric heat transport²⁸.

A critical factor in maintaining high-latitude warmth is maintaining ice-free conditions in polar oceans, conditions indicated by palaeobotanical evidence for near-freezing Arctic cold-month means²⁹ and the absence of sedimentological evidence for sea ice²⁶. Two proposed warming mechanisms are high concentrations of greenhouse gases¹ and increased efficiency of ocean heat transport combined with interior seaways^{30,31}. However, high concentrations of greenhouse gases can cause excessive tropical heating³², and how oceans can more efficiently transport heat is not well understood. High-latitude forest vegetation provides a third mechanism that contributed to high-latitude warmth during the Late Cretaceous and improves correlation of model climate and palaeovegetation. Thus, changes in land-surface characteristics by high-latitude forests, together with interaction between the land surface, atmosphere and ocean, may represent an important component of high-latitude warmth during warm geological periods. This emphasizes the potential role of extant high-latitude forests as important modifiers of anthropogenic climate change^{33–35}. □

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Supplementary Information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from Mary Sheehan at the London editorial office of Nature.

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Lower Palaeolithic hunting spears from Germany

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Little is known about the organic component of Lower and Middle Palaeolithic technologies, particular with respect to wooden tools^{1,2}. Here I describe some wooden throwing spears about 400,000 years old that were discovered in 1995 at the Pleistocene site at Schöningen, Germany. They are thought to be the oldest complete hunting weapons so far discovered to have been used by humans. Found in association with stone tools and the butchered remains of more than ten horses, the spears strongly suggest that systematic hunting, involving foresight, planning and the use of appropriate technology, was part of the behavioural repertoire of pre-modern hominids. The use of sophisticated spears as early as the Middle Pleistocene may mean that many current theories on early human behaviour and culture must be revised.

In 1983 I began archaeological excavations in the area of the Schöningen brown-coal mine. The mine complex has an area of $\sim 6\text{ km}^2$, of which more than $350,000\text{ m}^2$ has been excavated^{3,4}. During the course of the mining operation and excavation of Holocene sites, the Pleistocene exposures were constantly monitored and analysed^{5–7}.

The Schöningen brown-coal open-cast mine is situated in the northwestern part of central Europe, about 100 km east of Hannover, in the northern foreland of the Harz mountains, at the southeastern edge of the Triassic limestone ridge called the Elm^{3,8}. Since 1992, several Lower Palaeolithic sites have been discovered in Middle Pleistocene interglacial sediments 8–15 m below the surface⁸. The oldest Pleistocene deposits exposed in the mine are

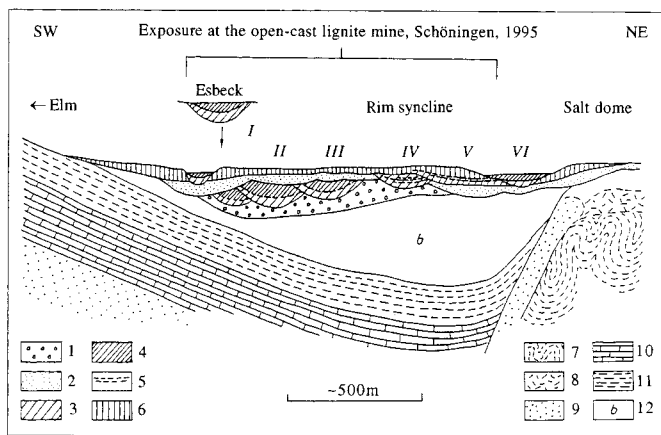


Figure 1 The brown-coal mine at Schöningen. A section through the cyclical Quaternary sedimentary sequence, in the depression along the Stassfurt-Helmstedter Salt dome⁸, is shown. The relative distance between cycle VI and the salt dome is reduced in this figure; the actual distance is about 2 km. The oldest interglacial sediments, Schöningen I, in the southern part of the pit, probably date from the Holsteinian (Esbeck). The second channel (Schöningen II) contains sediments of the Reinsdorf Interglacial (these deposits yielded the spears); palynological analysis indicates that its vegetational history differs from both the preceding Holsteinian and the following Schöningen Interglacials (Schöningen III), which is correlated to the Dömnitz Interglacial^{10,11} (Fig. 2). The Schöningen IV channel is younger than the Saale glacial *sensu stricto* (Drenthe), and consists of an extensive double soil complex. The infill of channel V is correlated to the Eemian Interglacial, whereas the sixth channel infill is of Holocene age. A correlation has been established between the Schöningen sequence and the terrace-travertine series at Bilzingsleben (Thuringia, Germany)¹²⁻¹⁴. 1, Elsterian glacial deposits; 2, Saalian glacial deposits; 3, lacustrine deposits; 4, limnic telmatic sequences; 5, soil complexes; 6, loess deposits; 7, evaporites; 8, gypsum cap-rock; 9, buntsandstein; 10, Triassic limestone (Muschelkalk); 11, Triassic deposits (Keuper); 12, Tertiary deposits¹³.

the sediments of the Elster glaciation (Fig. 1), above which a series of six major erosional channels have been identified. Multidisciplinary analysis of the channel deposits suggests that they range in age from the Holsteinian to the Holocene. The channels and their associated sediments represent a series of interglacial/glacial cycles that have been named Schöningen I–VI (Figs 1 and 2). Channels I–III, which contain limnic sediments, date to the period between the Elster and Saale glacial *sensu stricto* and cover three full interglacial/glacial cycles (Figs 1 and 2).

The oldest evidence of human occupation at the site is situated by a lake, and dates from the earliest part of the Holsteinian complex (Schöningen I) (Fig. 2). It comprises flint tools, flakes and numerous burnt flints, together with faunal remains of steppe elephant (*Mammuthus trogontherii*), bovinds, horse and red deer⁸ (Schöningen 13 I).

The Schöningen II channel (Fig. 1) is filled by sediments of the Reinsdorf Interglacial^{18,15} and the ensuing Fuhne cold stage (Fig. 2). The depositional sequence contains five levels of organic muds and peats (1–5). Level 1 (the bottom, oldest level) probably represents both the early and interglacial maxima of the Reinsdorf Interglacial. The upper levels represent cool temperate phases and exhibit frost structures between levels 4 and 5. The Reinsdorf Interglacial is a new biostratigraphical unit between the Elster and Saale *sensu stricto*^{10,11}.

Lake-shore deposits from the Reinsdorf Interglacial level 1 contained numerous flint artefacts and three worked branches of the common silver fir, *Abies alba*^{8,15,18}. The wooden tools (length 170–320 mm, maximum width 36–42 mm) have a diagonal groove cut

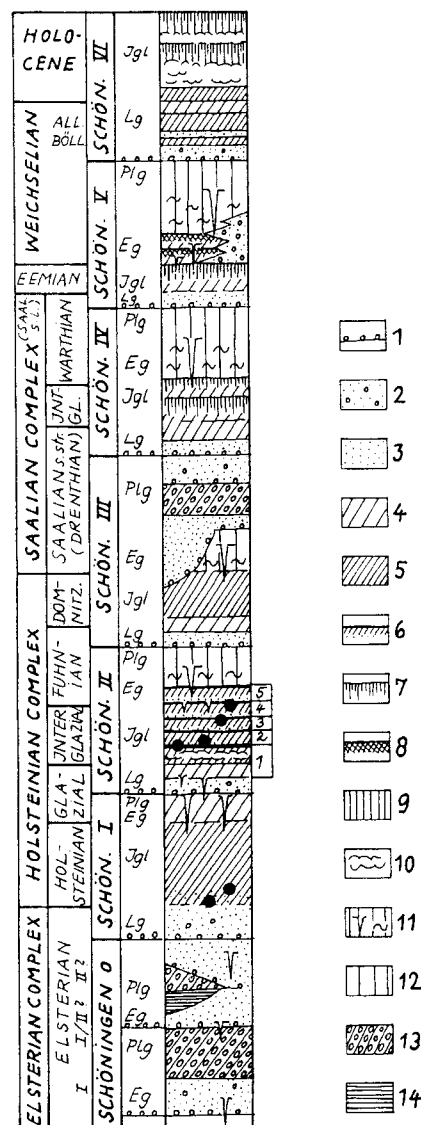


Figure 2 Composite schematic stratigraphical sequence through the Schöningen (Schön.) deposits, which cover the period from the Middle Pleistocene to the Holocene. Analysis of the *Arvicola* molars from Schöningen II, level 1 (the Reinsdorf Interglacial) suggests a correlation with the Bilzingsleben hominid site¹⁶⁻¹⁷. 1, Denudation horizon; 2, gravelly sands; 3, sands; 4, lacustrine deposits; 5, limnic organogenic sediments; 6, peat; 7, soils (lessivé, pseudogley); 8, humic zones; 9, fine-grained alluvial deposits; 10, travertine; 11, periglacial structures; 12, loess; 13, ground moraines; 14, laminated clay deposits. Black dots indicate archaeological find horizons. The spears are from level 4 within the Schöningen II sequence, and date from the end of the Reinsdorf Interglacial. Lg, late glacial; Plg, pleniglacial; early glacial; Igl, interglacial. 1–5 indicate upward shallowing sequences in the Reinsdorf Interglacial.

into one end. The grooves vary in length between 33 and 47 mm in the front, and 10 to 30 mm at the back; groove breadth varies between 4 and 9 mm at the distal end. It is postulated that the grooves were for holding flint tools or flakes; if this supposition is correct, these implements represent the oldest composite tools yet discovered. Associated with these finds were more than a thousand large mammal bones, some of which exhibit cut marks from butchery. The fauna includes straight-tusked elephant *Palaeoloxodon antiquus*, rhinoceros *Stephanorhinus kirchbergensis*, red deer *Cervus elaphus*, bear and horse. There are also numerous small mammals,

Table 1 Wooden implements from Schöningen 13 II-4 (channel II, level 4)

Implement	Length (m)	Tip length (mm)	Maximum diameter (mm)	Point of maximum diameter* (mm)	Midpoint thickness (mm)	Thickness at 1/3 length* (mm)	Thickness at 2/3 length* (mm)
Spear I	2.25	>600	47	700	36	47	35
Spear II	2.30	>600	37	800	35	37	34
Spear III	1.82	>250	29	300	24	27	23
Throwing stick?	0.78	140 120	30	260	27	30	23

These data are preliminary: the wood has been slightly deformed owing to sediment pressure and the influence of permafrost. Asterisks indicate measurements taken from the distal end of the spear.

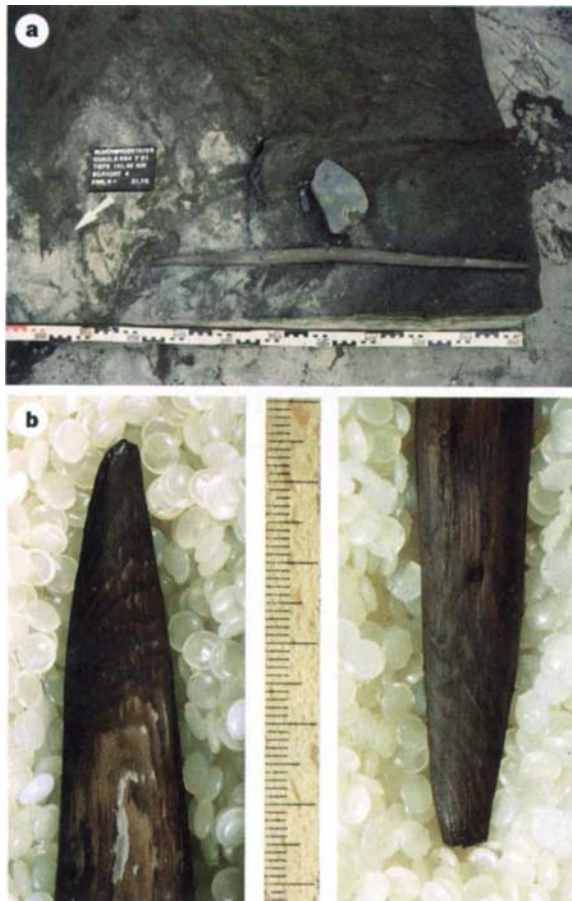


Figure 3 a, View of the throwing stick (?) in the field. Close to the stick is a larger bone fragment, on the left of which is a flint scraper. The scale is calibrated in cm. **b**, The pointed tips of the throwing stick (?). One tip (right) was slightly damaged during excavation. Scale in cm.



Figure 4 Spear I during excavation. Postsedimentary processes led to fragmentation of the spear in five parts: 1 (base) to 5 (tip). Bones are also visible, with one long bone on top of fragment 4.

faunal remains, mainly horse, from an area of 1,200 m² (in autumn 1996), with densities of up to 100 pieces per square metre (Schöningen 13 II-4). Many of the bones display traces of butchery, in the form of cut marks and fracturing. The stone artefacts were all made of flint, including some points, carefully retouched scrapers, and hundreds of spalls. In the southwestern part of the excavation a possible hearth was excavated; the chalky sediments in this area became red under the influence of the heat of the fire, and display many fossil drying cracks.

The wooden tools from level 4 comprise an implement of indeterminate function (a thrusting spear or a throwing stick?) that has both ends sharpened to a point (Fig. 3 and Table 1) and three spears (Figs 4 and 5 and Table 1). All of these are made from spruce *Picea* sp. (ref. 8) and were found in conjunction with abundant faunal remains. The wood selected exhibits a dense concentration of growth rings, implying slow growing conditions in a cool environment. The spears are made from individual trees, which were felled, and the branches and bark were removed; the tip/distal ends are worked from the base of the three (Fig. 5). All three spears, although of different lengths, were manufactured to the same pattern, with the maximum thickness and weight at the front (Table 1); the tails are long, and taper towards the proximal end. In all of these respects they resemble modern javelins, and were made as projectile weapons rather than thrusting spears or lances.

These finds are thought to be the oldest wooden throwing spears yet discovered. As they are sandwiched between deposits of the Elsterian and the Saalian glaciations, and situated within a well-studied sedimentary sequence, their age can be estimated as probably 400 kyr. Studies of the litho-, pedo- and biostratigraphy of the pit have shown that the sediments of the newly discovered Reinsdorf Interglacial date from a temperate period that is older than oxygen-

including the water vole *Arvicola terrestris cantiana* and the extinct, beaver-like *Trogontherium cuvieri*, together with the remains of birds, fish and reptiles (Schöningen 12, find layer 1).

Further archaeological material has been recovered from level 4 of the Schöningen II channel (Figs 1 and 2), in an organic mud that underlies a peat horizon. Preliminary analyses of the molluscan fauna found there and the pollen spectra suggest a boreal, cool-temperate climate; the vegetation indicates a mix of meadows and forest steppes with an abundance of pine, spruce and birch woodland. The excavation yielded more than 10,000 well-preserved



Figure 5 Spear II, which is 2.30 m long. The spear is shown to the left of an incomplete pelvis of a horse, and the base has been broken off. Inset shows a detail of the tip of spear II. Scale in cm.

isotope stage 7, and are at least of stage 9. Correlations of the Schöningen sequence to other areas^{12,14} suggest that they were deposited during the fourth-last interglacial, probably at the end of stage 11. Judging from the mammalian fauna, the Reinsdorf assemblage is younger than the Lower Palaeolithic site of Boxgrove (West Sussex, UK)^{19,20}.

Before our discovery, the oldest complete 'spear' known was discovered in Eemian deposits at Lehringen (Lower Saxony, Germany) in 1948 (ref. 2). Thought to be about 125 kyr old (oxygen-isotope substage 5e), this thrusting spear was recovered from between the ribs of a straight-tusked elephant, and was made from yew (*Taxus*). The Schöningen spears are probably three full interglacial/glacial cycles older than the Lehringen lance (Fig. 2).

The discovery of spears designed for throwing means that theories of the development of hunting capacities and subsistence strategies of Middle Pleistocene hominids must be revised, as well-balanced, sophisticated hunting weapons were common from an early period of the Middle Pleistocene onwards. Accordingly, meat from hunting may have provided a larger dietary contribution than has previously been acknowledged^{21,22}. The Schöningen evidence also illustrates how little is known about the 'organic' component of early hominid material culture. □

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Viable offspring derived from fetal and adult mammalian cells

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Fertilization of mammalian eggs is followed by successive cell divisions and progressive differentiation, first into the early embryo and subsequently into all of the cell types that make up the adult animal. Transfer of a single nucleus at a specific stage of development, to an enucleated unfertilized egg, provided an opportunity to investigate whether cellular differentiation to that stage involved irreversible genetic modification. The first offspring to develop from a differentiated cell were born after nuclear transfer from an embryo-derived cell line that had been induced to become quiescent¹. Using the same procedure, we now report the birth of live lambs from three new cell populations established from adult mammary gland, fetus and embryo. The fact that a lamb was derived from an adult cell confirms that differentiation of that cell did not involve the irreversible modification of genetic material required for development to term. The birth of lambs from differentiated fetal and adult cells also reinforces previous speculation^{1,2} that by inducing donor cells to become quiescent it will be possible to obtain normal development from a wide variety of differentiated cells.

It has long been known that in amphibians, nuclei transferred from adult keratinocytes established in culture support development to the juvenile, tadpole stage³. Although this involves differentiation into complex tissues and organs, no development to the adult stage was reported, leaving open the question of whether a differentiated adult nucleus can be fully reprogrammed. Previously we reported the birth of live lambs after nuclear transfer from cultured embryonic cells that had been induced into quiescence. We suggested that inducing the donor cell to exit the growth phase

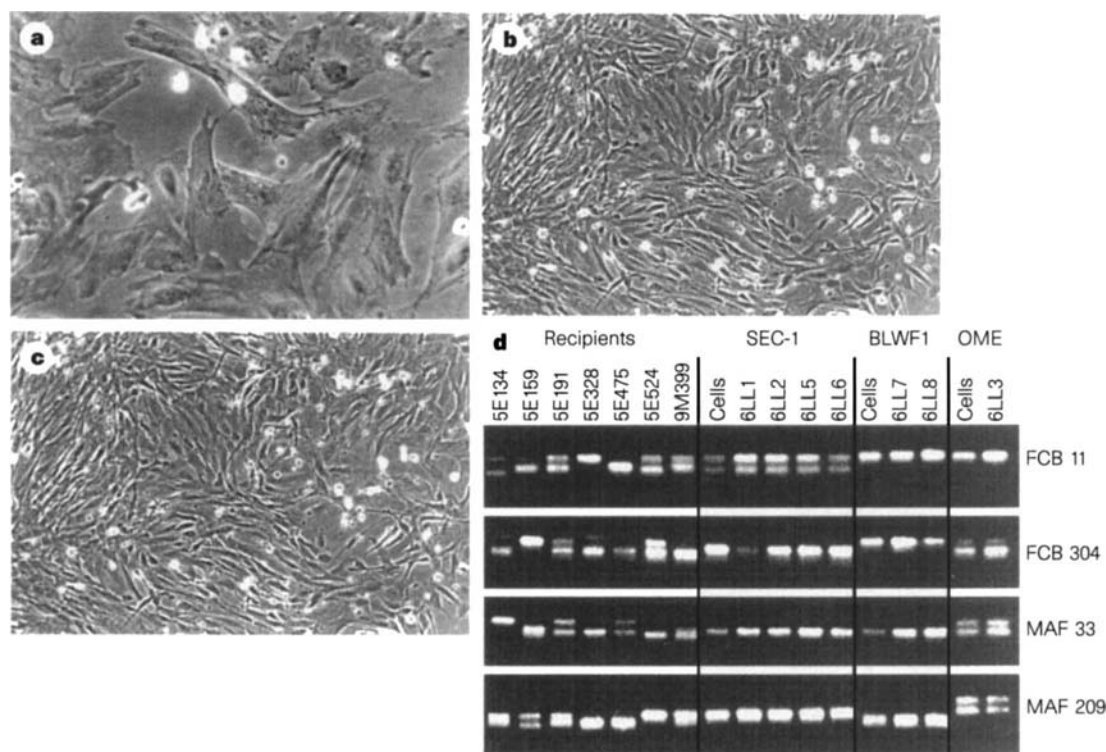


Figure 1 Phase-contrast photomicrograph of donor-cell populations: **a**, Embryo-derived cells (SEC1); **b**, fetal fibroblasts (BLWF1); **c**, mammary-derived cells (OME). **d**, Microsatellite analysis of recipient ewes, nuclear donor cells and lambs using four polymorphic ovine markers²². The ewes are arranged from left to right

in the same order as the lambs. Cell populations are embryo-derived (SEC1), fetal-derived (BLWF1), and mammary-derived (OME), respectively. Lambs have the same genotype as the donor cells and differ from their recipient mothers.

causes changes in chromatin structure that facilitate reprogramming of gene expression and that development would be normal if nuclei are used from a variety of differentiated donor cells in similar regimes. Here we investigate whether normal development to term is possible when donor cells derived from fetal or adult tissue are induced to exit the growth cycle and enter the G0 phase of the cell cycle before nuclear transfer.

Three new populations of cells were derived from (1) a day-9 embryo, (2) a day-26 fetus and (3) mammary gland of a 6-year-old ewe in the last trimester of pregnancy. Morphology of the embryo-derived cells (Fig. 1) is unlike both mouse embryonic stem (ES) cells and the embryo-derived cells used in our previous study. Nuclear transfer was carried out according to one of our established protocols¹ and reconstructed embryos transferred into recipient ewes. Ultrasound scanning detected 21 single fetuses on day 50–60 after oestrus (Table 1). On subsequent scanning at ~14-day intervals, fewer fetuses were observed, suggesting either mis-diagnosis or

fetal loss. In total, 62% of fetuses were lost, a significantly greater proportion than the estimate of 6% after natural mating⁴. Increased prenatal loss has been reported after embryo manipulation or culture of unreconstructed embryos⁵. At about day 110 of pregnancy, four fetuses were dead, all from embryo-derived cells, and post-mortem analysis was possible after killing the ewes. Two fetuses had abnormal liver development, but no other abnormalities were detected and there was no evidence of infection.

Eight ewes gave birth to live lambs (Table 1, Fig. 2). All three cell populations were represented. One weak lamb, derived from the fetal fibroblasts, weighed 3.1 kg and died within a few minutes of birth, although post-mortem analysis failed to find any abnormality or infection. At 12.5%, perinatal loss was not dissimilar to that occurring in a large study of commercial sheep, when 8% of lambs died within 24 h of birth⁶. In all cases the lambs displayed the morphological characteristics of the breed used to derive the nucleus donors and not that of the oocyte donor (Table 2). This

Table 1 Development of embryos reconstructed with three different cell types

Cell type	No. of fused couplets (%) ^a	No. recovered from oviduct (%)	No. cultured	No. of morula/blastocyst (%)	No. of morula or blastocysts transferred [†]	No. of pregnancies/no. of recipients (%)	No. of live lambs (%) [‡]
Mammary epithelium	277 (63.8) ^a	247 (89.2)	–	29 (11.7) ^a	29	1/13 (7.7)	1 (3.4%)
Fetal fibroblast	172 (84.7) ^b	124 (86.7)	–	34 (27.4) ^b	34	4/10 (40.0)	2 (5.9%)
			24	13 (54.2) ^b	6	1/6 (16.6)	1 (16.6%) [§]
Embryo-derived	385 (82.8) ^b	231 (85.3)	–	90 (39.0) ^b	72	14/27 (51.8)	4 (5.6%)
			92	36 (39.0) ^b	15	1/5 (20.0)	0

^a As assessed 1 h after fusion by examination on a dissecting microscope. Superscripts a or b within a column indicate a significant difference between donor cell types in the efficiency of fusion ($P < 0.001$) or the proportion of embryos that developed to morula or blastocyst ($P < 0.001$).

[†] It was not practicable to transfer all morulae/blastocysts.

[‡] As a proportion of morulae or blastocysts transferred. Not all recipients were perfectly synchronized.

[§] This lamb died within a few minutes of birth.



Figure 2 Lamb number 6LL3 derived from the mammary gland of a Finn Dorset ewe with the Scottish Blackface ewe which was the recipient.

alone indicates that the lambs could not have been born after inadvertent mating of either the oocyte donor or recipient ewes. In addition, DNA microsatellite analysis of the cell populations and the lambs at four polymorphic loci confirmed that each lamb was derived from the cell population used as nuclear donor (Fig. 1). Duration of gestation is determined by fetal genotype⁷, and in all cases gestation was longer than the breed mean (Table 2). By contrast, birth weight is influenced by both maternal and fetal genotype⁸. The birth weight of all lambs was within the range for single lambs born to Blackface ewes on our farm (up to 6.6 kg) and in most cases was within the range for the breed of the nuclear donor. There are no strict control observations for birth weight after embryo transfer between breeds, but the range in weight of lambs born to their own breed on our farms is 1.2–5.0 kg, 2–4.9 kg and 3–9 kg for the Finn Dorset, Welsh Mountain and Poll Dorset genotypes, respectively. The attainment of sexual maturity in the lambs is being monitored.

Development of embryos produced by nuclear transfer depends upon the maintenance of normal ploidy and creating the conditions for developmental regulation of gene expression. These responses are both influenced by the cell-cycle stage of donor and recipient cells and the interaction between them (reviewed in ref. 9). A comparison of development of mouse and cattle embryos produced by nuclear transfer to oocytes^{10,11} or enucleated zygotes^{12,13} suggests that a greater proportion develop if the recipient is an oocyte. This may be because factors that bring about reprogramming of gene expression in a transferred nucleus are required for early development and are taken up by the pronuclei during development of the zygote.

If the recipient cytoplasm is prepared by enucleation of an oocyte at metaphase II, it is only possible to avoid chromosomal damage and maintain normal ploidy by transfer of diploid nuclei^{14,15}, but further experiments are required to define the optimum cell-cycle stage. Our studies with cultured cells suggest that there is an advantage if cells are quiescent (ref. 1, and this work). In earlier studies, donor cells were embryonic blastomeres that had not been induced into quiescence. Comparisons of the phases of the growth cycle showed that development was greater if donor cells were in mitosis¹⁶ or in the G1 (ref. 10) phase of the cycle, rather than in S or G2 phases. Increased development using donor cells in G0, G1 or mitosis may reflect greater access for reprogramming factors present in the oocyte cytoplasm, but a direct comparison of these phases in the same cell population is required for a clearer understanding of the underlying mechanisms.

Table 2 Delivery of lambs developing from embryos derived by nuclear transfer from three different donor cell types, showing gestation length and birth weight

Cell type	Breed of lamb	Lamb identity	Duration of pregnancy (days)*	Birth weight (kg)
Mammary epithelium	Finn Dorset	6LL3	148	6.6
Fetal fibroblast	Black Welsh	6LL7	152	5.6
	Black Welsh	6LL8	149	2.8
	Black Welsh	6LL9†	156	3.1
Embryo-derived	Poll Dorset	6LL1	149	6.5
	Poll Dorset	6LL2‡	152	6.2
	Poll Dorset	6LL5	148	4.2
	Poll Dorset	6LL6‡	152	5.3

* Breed averages are 143, 147 and 145 days, respectively for the three genotypes Finn Dorset, Black Welsh Mountain and Poll Dorset.

† This lamb died within a few minutes of birth.

‡ These lambs were delivered by caesarian section. Overall the nature of the assistance provided by the veterinary surgeon was similar to that expected in a commercial flock.

Together these results indicate that nuclei from a wide range of cell types should prove to be totipotent after enhancing opportunities for reprogramming by using appropriate combinations of these cell-cycle stages. In turn, the dissemination of the genetic improvement obtained within elite selection herds will be enhanced by limited replication of animals with proven performance by nuclear transfer from cells derived from adult animals. In addition, gene targeting in livestock should now be feasible by nuclear transfer from modified cell populations and will offer new opportunities in biotechnology. The techniques described also offer an opportunity to study the possible persistence and impact of epigenetic changes, such as imprinting and telomere shortening, which are known to occur in somatic cells during development and senescence, respectively.

The lamb born after nuclear transfer from a mammary gland cell is, to our knowledge, the first mammal to develop from a cell derived from an adult tissue. The phenotype of the donor cell is unknown. The primary culture contains mainly mammary epithelial (over 90%) as well as other differentiated cell types, including myoepithelial cells and fibroblasts. We cannot exclude the possibility that there is a small proportion of relatively undifferentiated stem cells able to support regeneration of the mammary gland during pregnancy. Birth of the lamb shows that during the development of that mammary cell there was no irreversible modification of genetic information required for development to term. This is consistent with the generally accepted view that mammalian differentiation is almost all achieved by systematic, sequential changes in gene expression brought about by interactions between the nucleus and the changing cytoplasmic environment¹⁷. □

Methods

Embryo-derived cells were obtained from embryonic disc of a day-9 embryo from a Poll Dorset ewe cultured as described¹, with the following modifications. Stem-cell medium was supplemented with bovine DIA/LIF. After 8 days, the explanted disc was disaggregated by enzymatic digestion and cells replated onto fresh feeders. After a further 7 days, a single colony of large flattened cells was isolated and grown further in the absence of feeder cells. At passage 8, the modal chromosome number was 54. These cells were used as nuclear donors at passages 7–9. Fetal-derived cells were obtained from an eviscerated Black Welsh Mountain fetus recovered at autopsy on day 26 of pregnancy. The head was removed before tissues were cut into small pieces and the cells dispersed by exposure to trypsin. Culture was in BHK 21 (Glasgow MEM; Gibco Life Sciences) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM) and 10% fetal calf serum. At 90% confluency, the cells were passaged with a 1 : 2

division. At passage 4, these fibroblast-like cells (Fig. 1) had modal chromosome number of 54. Fetal cells were used as nuclear donors at passages 4–6. Cells from mammary gland were obtained from a 6-year-old Finn Dorset ewe in the last trimester of pregnancy¹⁸. At passages 3 and 6, the modal chromosome number was 54 and these cells were used as nuclear donors at passage numbers 3–6.

Nuclear transfer was done according to a previous protocol¹. Oocytes were recovered from Scottish Blackface ewes between 28 and 33 h after injection of gonadotropin-releasing hormone (GnRH), and enucleated as soon as possible. They were recovered in calcium- and magnesium-free PBS containing 1% FCS and transferred to calcium-free M2 medium¹⁹ containing 10% FCS at 37 °C. Quiescent, diploid donor cells were produced by reducing the concentration of serum in the medium from 10 to 0.5% for 5 days, causing the cells to exit the growth cycle and arrest in G0. Confirmation that cells had left the cycle was obtained by staining with antiPCNA/cyclin antibody (Immuno Concepts), revealed by a second antibody conjugated with rhodamine (Dakopatts).

Fusion of the donor cell to the enucleated oocyte and activation of the oocyte were induced by the same electrical pulses, between 34 and 36 h after GnRH injection to donor ewes. The majority of reconstructed embryos were cultured in ligated oviducts of sheep as before, but some embryos produced by transfer from embryo-derived cells or fetal fibroblasts were cultured in a chemically defined medium²⁰. Most embryos that developed to morula or blastocyst after 6 days of culture were transferred to recipients and allowed to develop to term (Table 1). One, two or three embryos were transferred to each ewe depending upon the availability of embryos. The effect of cell type upon fusion and development to morula or blastocyst was analysed using the marginal model of Breslow and Clayton²¹. No comparison was possible of development to term as it was not practicable to transfer all embryos developing to a suitable stage for transfer. When too many embryos were available, those having better morphology were selected.

Ultrasound scan was used for pregnancy diagnosis at around day 60 after oestrus and to monitor fetal development thereafter at 2-week intervals. Pregnant recipient ewes were monitored for nutritional status, body condition and signs of EAE, Q fever, border disease, louping ill and toxoplasmosis. As lambing approached, they were under constant observation and a veterinary surgeon called at the onset of parturition. Microsatellite analysis was carried out on DNA from the lambs and recipient ewes using four polymorphic ovine markers²².

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Evidence against a dedicated system for word learning in children

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Children can learn aspects of the meaning of a new word on the basis of only a few incidental exposures and can retain this knowledge for a long period—a process dubbed ‘fast mapping’^{1–8}. It is often maintained that fast mapping is the result of a dedicated language mechanism, but it is possible that this same capacity might apply in domains other than language learning. Here we present two experiments in which three- and four-year-old children and adults were taught a novel name and a novel fact about an object, and were tested on their retention immediately, after a 1-week delay or after a 1-month delay. Our findings show that fast mapping is not limited to word learning, suggesting that the capacity to learn and retain new words is the result of learning and memory abilities that are not specific to language.

In two experiments (study 1 and study 2), 48 three-year-old children (mean age, 3 yr 7 months), 47 four-year-old children (mean age, 4 yr 5 months) and 48 undergraduate students first participated in a training phase that lasted for about twenty minutes. This phase involved the manipulation of ten kinds of objects, four of them familiar (for example, pennies) and six of them novel (see Methods). Subjects were asked to use some of the objects to measure other objects: for instance, they were asked to use pennies to measure the circumference of a plastic disc. Children were told it was a game, and adults were told it was a game designed to teach young children how to measure.

In the course of the training phase, subjects in both study 1 and study 2 were exposed to a new word—‘koba’—used to refer to one of the six unfamiliar kinds of objects. Subjects in study 1 were also taught a new fact about one or more objects belonging to another kind. They were told that the object or objects was given to the experimenter by her uncle. Subjects in study 2 were given information about an unfamiliar object, presented visually. They watched as a sticker was placed on one of the unfamiliar objects, and were told that was where the sticker should go (see Methods).

In each of the studies, one-third of the subjects from each age group were tested for comprehension immediately after the training phase, one-third were tested after a 1-week delay (6–8 days), and one-third after a 1-month delay (28–30 days). Subjects were presented with the original array of ten items and asked to recall which object was the koba. Subjects in study 1 were also asked to recall which object was given to the experimenter by her uncle. Subjects in study 2 were handed a small sticker and instructed to put it where it should go (see Methods).

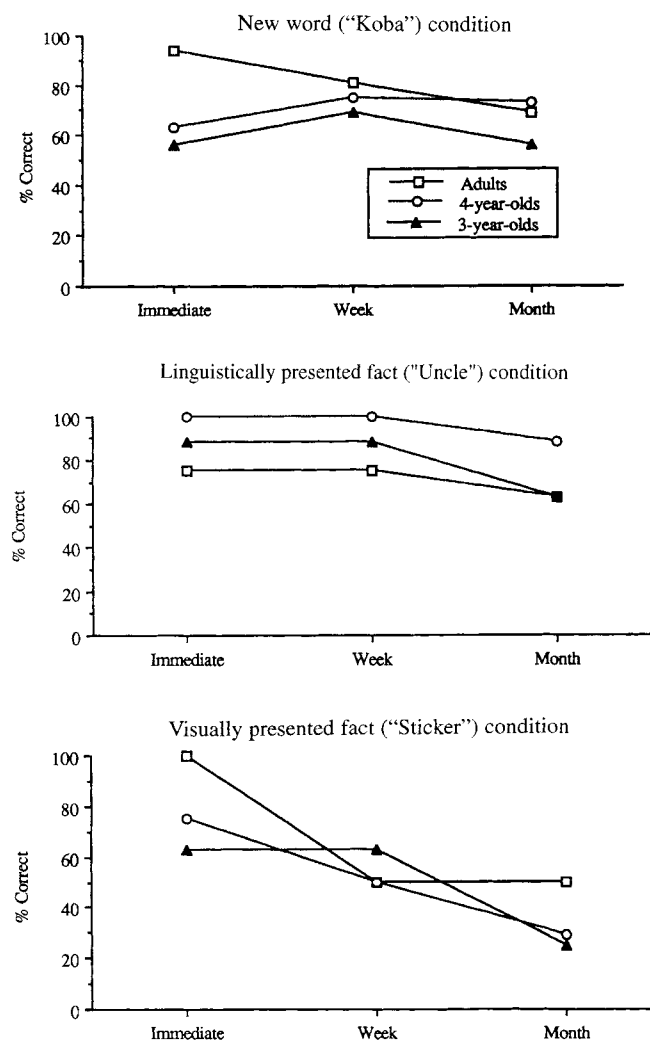


Figure 1 Percentage of subjects who recalled the object to which the novel word referred, the object that had the property of being given to the experimenter by her uncle, and the object that had a sticker affixed to it. 'Koba' condition: Adults were significantly better than both the three- and four-year-olds when tested immediately (adults versus three-year-olds, $\chi^2(1) = 6.0$; adults versus four-year-olds, $\chi^2(1) = 4.6$, both P values of <0.05). Unlike the two conditions below, in which chance performance was calculated as $1/10$ (as there were ten objects), chance performance was calculated as $1/6$, because four of the ten objects were familiar, and children and adults assume that novel words are more likely to correspond to novel objects than to familiar ones¹⁵. As a result, even if subjects forgot which object the new word referred to, they might restrict their guess to the six novel objects. Based on these criteria, all ages performed significantly better than chance in all delay conditions. Also, the 3-year-olds performed significantly better when the new word was presented in the singular rather than in the plural form ($P < 0.01$); this was the only effect of the singular/plural distinction. 'Uncle' condition: All age groups again performed significantly better than chance in all delay conditions. When the three- and four-year-olds were combined into a single group, they did significantly better in the 'Uncle' condition than in the 'Koba' condition when tested immediately ($\chi^2(1) = 6.1$, $P < 0.05$). 'Sticker' condition: Adults did significantly better when tested immediately than when tested after a week and when tested after a month (Fisher's exact tests, $P = 0.04$). the three- and four-year-olds' performance was significantly worse after a one-week delay than it was in the 'Uncle' condition, and significantly worse after a one-month delay than it was in both the 'Koba' and the 'Uncle' conditions. Only the adults performed significantly better than chance after a one-month delay; the three- and four-year-olds declined over time, doing significantly worse after a month than when tested immediately (all P values <0.05).

Children and adults successfully learned the novel object to which the label 'koba' referred, and were able to remember the correct word-object mapping after all tested delays (Fig. 1). Adults were significantly better than the three- and four-year-olds when tested immediately, but there were no age differences at the longer intervals, and no significant decline in performance over time by any of the age groups.

Interestingly, however, subjects were equally good at remembering which novel object was 'given to the experimenter by her uncle', suggesting that fast mapping is not special to word learning. In fact, when tested immediately, children did significantly better in this condition than in the word-learning condition. In contrast to the 'koba' and 'uncle' conditions, both children and adults were considerably worse at learning and remembering which object had a sticker affixed to it. All age groups performed significantly better when tested immediately than when tested after a delay. The children performed significantly worse in the 'sticker' condition than in the other two conditions, and only the adults in this condition performed significantly better than chance after a one-month delay; the children's performance after one month was indistinguishable from guessing.

One objection to the conclusion that fast mapping is not special to words is that the 'koba' condition requires subjects to learn and store a novel phonological representation, whereas the 'uncle' condition does not. It is possible that if both tasks involved a novel phonological form, subjects would do significantly worse at the non-word learning task. To test this, 15 three-year-olds (mean age,

3 yr 7 months), 15 four-year-olds (mean age, 4 yr 3 months) and 15 undergraduate students participated in a training phase in which they were taught an arbitrary fact that included an unfamiliar word. Specifically, subjects were taught that one of the unfamiliar kinds of objects 'came from a place called Koba'. They were tested on their memory of the new fact after a 1-month delay by being asked to recall which object 'came from a place called Koba'.

Children and adults performed significantly above chance in this condition, and there were no significant age differences (3-year-olds: 87%; 4-year-olds: 67%; adults: 60%). In fact, after this one-month delay, there were no significant differences between subjects' ability to recall which object 'came from a place called Koba' and their performance in the 'koba' and 'uncle' conditions. The fact that fast mapping still occurred when subjects had to learn a fact that contained a novel word alleviates the concern about task complexity.

In summary, children and adults were as good at learning and remembering an arbitrary linguistically presented fact about an entity as they were at remembering its name, even if the arbitrary fact contained a novel word. Nevertheless, fast mapping does not apply to any arbitrary memorization task, as illustrated by the children who, after a 1-month delay, were unable to recall the location of a sticker. This raises the question of the scope of this process. One possibility is that it applies only to information conveyed through language. Another is that salience is a factor, and that the children and adults simply found the source of the object to be a more interesting fact than the placement of the sticker,

and hence easier to remember. A third possibility is that fast mapping only applies in circumstances in which object identity or object category is considered relevant, as opposed to a property such as location. Nevertheless, although the precise scope of fast mapping remains a topic for further investigation, it is not limited to word learning.

In addition, we found no critical period for fast mapping; there was no advantage for children, at least for the time intervals studied here. In contrast, children are much better at learning phonology, morphology and syntax than adults⁹, consistent with the notion of a biological specialization for these aspects of language^{10,11}. In this regard, then, word learning is different from other aspects of language development. This difference also surfaces in developmental disorders in which word learning is intact but other aspects of language are severely impaired¹², as well as in event-related brain potential studies which find that lexical information is processed differently from grammatical information in both children and adults¹³. The finding that fast mapping is not restricted to word learning is therefore consistent with evidence from different sources which suggests that word learning is mediated by processes of human learning and memory that are not special to the domain of language¹⁴. □

Methods

The novel objects were roughly equal in size and were selected according to the criterion that subjects would be unable to name them. They included a white plastic rectangle consisting of ten plastic bars connected by two longer bars, a grey plastic grid, a wooden stick with a black sponge tip, a trumpet-shaped piece of pasta, a blue plastic tube with a ridged surface, and a brown rubber disc that was smooth on one side and indented with various circles on the other. The familiar objects included pennies, a pencil, a ruler and some string.

For half of the subjects in each study, the new word 'koba' was used in the singular to describe a single object; for the rest, it was used to describe multiple identical objects of that kind. Subjects were told either 'Let's measure the koba. We can count these to see how long the koba is. We can put the koba away now.' or 'Let's use the kobas to measure which is longer. Line up the kobas so we can count them. We can put the kobas away now.'

Subjects who were taught a singular label learned a fact about multiple objects of the same kind, whereas subjects who were taught a plural label for multiple objects of the same kind were taught a fact about a single object. In study 1, subjects were told: 'We can use the thing(s) my uncle gave to me to measure which is longer. My uncle gave these to me. We can put the thing(s) my uncle gave to me away now.' Subjects in study 2 watched as a sticker was placed on one of the unfamiliar objects, and were told: 'Watch where this goes. This goes here [placing the sticker onto one of the objects]. That's where this goes.' The specific objects presented in the different conditions were counterbalanced across subjects.

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A cGMP-gated cation channel in depolarizing photoreceptors of the lizard parietal eye

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Rods and cones of the two vertebrate lateral eyes hyperpolarize when illuminated, a response generated by a cyclic GMP cascade leading to cGMP hydrolysis and consequently the closure of cGMP-gated, non-selective cation channels that are open in darkness^{1–4}. Lizards and other lower vertebrates also have a parietal (third) eye⁵, which contains ciliary photoreceptors that under dark-adapted conditions depolarize to light instead⁶. Depolarizing light responses are characteristic of most invertebrate rhabdomeric photoreceptors, and are thought to involve a phosphoinositide signalling pathway (see, for example, refs 7–9). Surprisingly, we have found in excised membrane patches a cGMP-gated channel that is selectively present at high density on the outer segment (the presumptive light-sensitive part) of the parietal eye photoreceptor. Like the light-activated channel of the cell, it is non-selective among cations. Inositol trisphosphate (InsP₃) had no effect on the same membrane patches. These findings suggest that the photoreceptors of the parietal eye, like rods and cones, use a cGMP cascade and not an InsP₃-mediated pathway for phototransduction, but in this case light increases cGMP. A unifying principle of evolutionary significance emerges: that phototransductions in various ciliary photoreceptors, whether hyperpolarizing or depolarizing, uniformly use a cGMP cascade and a cGMP-gated channel to generate the light response, although there are rich variations in the details.

We recorded under voltage clamp from inside-out membrane patches excised from the outer segments of dissociated parietal-eye photoreceptors of the side-blotched lizard, *Uta stansburiana*. These photoreceptors are remarkably similar to rods and cones in morphology (Fig. 1a). The outer segment of the photoreceptor, like the cone outer segment, is composed of a continuous sheet of convoluted plasma membrane⁵. Bath-applied cGMP elicited a pronounced membrane current from most excised patches (85 out of 120; the rest might have been sealed-off vesicles); cAMP was much less effective (Fig. 1b, c). The current could be activated repeatedly by cyclic nucleotides without ATP, suggesting a direct effect. The blocker *l*-cis-diltiazem inhibited the channel with a half-maximal inhibitory concentration (IC₅₀) of ~3 μM at +60 mV (data not shown). Ca²⁺-calmodulin reduced the current maximally by about 50% by shifting the half-activation constant, K_{1/2} (Fig. 1d). Overall, the channel's activation and modulation properties are similar to those of the rod cGMP-gated channel¹⁰.

The depolarizing response to light of the dark-adapted parietal-eye photoreceptor is associated with an increase in membrane conductance and a reversal potential near zero (ref. 6); thus the phototransducing channel appears non-selective among cations. For the cGMP-gated channel, changing the bath NaCl concentration in our experiments from initially symmetrical NaCl solutions shifted the reversal potential, V_{rev}, in a Nernstian manner expected of a cation channel (Fig. 1e). From the respective shift in V_{rev} when all bath Na⁺ was replaced with Li⁺, K⁺, Rb⁺ or Cs⁺ (Fig. 1f), the estimated permeability ratios P_{Li} : P_{Na} : P_K : P_{Rb} : P_{Cs} were 1.23 : 1 : 0.83 : 0.58 : 0.39 (average, 4 experiments). Thus the channel is relatively non-selective among monovalent cations.

With 2 mM Ca^{2+} and 1 mM Mg^{2+} extracellularly (in the pipette), the current–voltage relation was strongly outward-rectifying (Fig. 2a), unlike the near-linear relation without divalent cations (Fig. 1f). Thus the channel is blocked by divalent cations in a similar way to the rod channel¹⁰. To test for the permeability of divalent cations, we replaced all bath Na^+ with divalent cations¹¹. At positive voltages there was an outward current that could only be carried by divalent cations (Fig. 2b). The Ca^{2+} permeability was measured by adding bath CaCl_2 to symmetrical NaCl solutions¹². Ca^{2+} reduced the macroscopic current in a way expected for a permeant blocker^{11–13} (Fig. 2c, e; note the gradual relief of block at increasingly positive voltages in e), and shifted V_{rev} to more negative voltages (Fig. 2d). The dependence of V_{rev} on Ca^{2+} activity (Fig. 2f) gave a high permeability

ratio, $P_{\text{Ca}}/P_{\text{Na}}$ of 10.3 (Fig. 2 legend), similar to that for the rod channel measured under identical conditions (ref. 12, and A. Picones, personal communication). Whether this channel is indeed identical to the rod channel, or represents a new member of the family of cGMP-gated channels, can only be determined by molecular cloning.

If the cGMP-gated channel is specifically involved in phototransduction, it should be present exclusively, or at a much higher density, on the outer segment. Experiments to estimate this density are shown in Fig. 3. The maximum current elicited by cGMP, I_{max} , from an excised patch is given by $I_{\text{max}} = NP_o i$, where N is the number of channels in the patch, P_o is the open probability of the liganded channel, and i is the single-channel current. At +60 mV, the single-channel current induced by cGMP in the

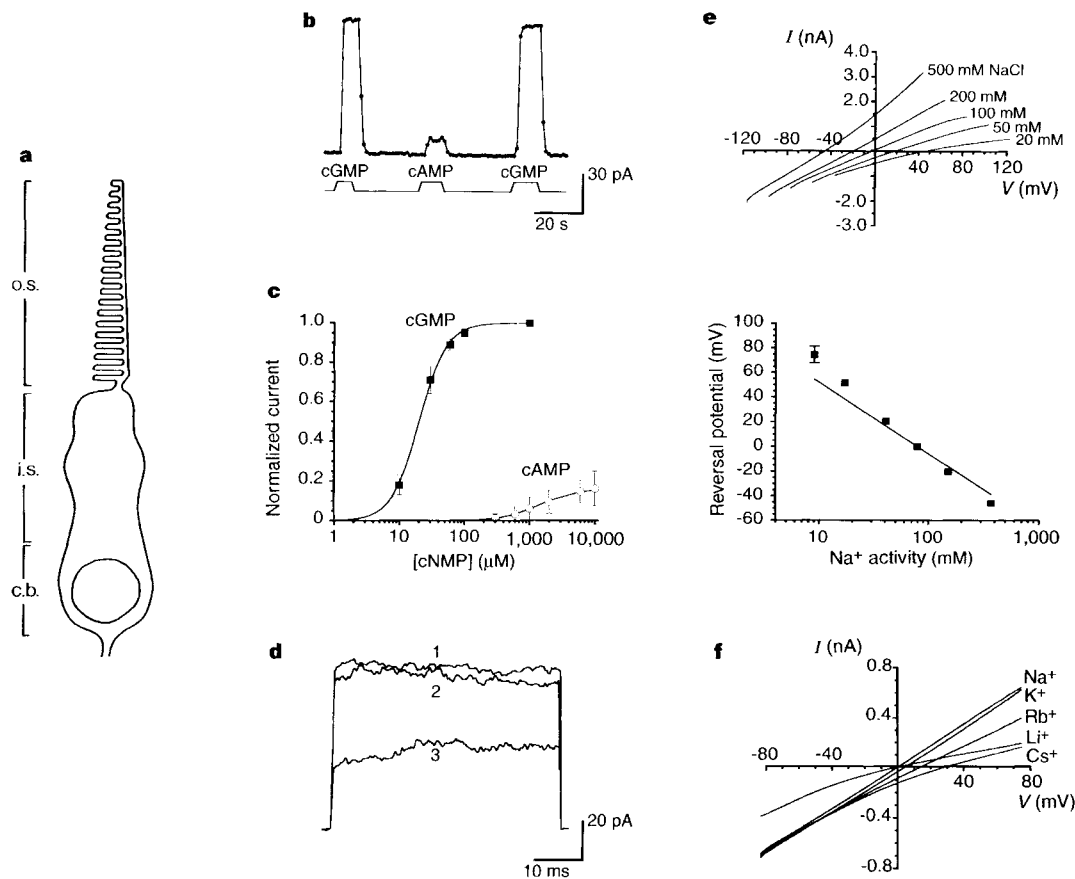


Figure 1 **a**, Diagram of a parietal-eye photoreceptor of the lizard; o.s., outer segment; i.s., inner segment; c.b., cell body. The density of membranous discs is ~25–30 per μm . The outer segment can be up to $10\mu\text{m}$ in length. **b**, Cyclic nucleotide-activated currents recorded from an excised, inside-out patch of outer-segment membrane of a lizard parietal-eye photoreceptor. Symmetrical Na^+ solutions without divalent cations (see Methods); 1 mM cGMP or cAMP. Each point represents the average current in a 50-ms step from 0 to +60 mV given at 1 Hz. Leakage current in the absence of cyclic nucleotide was already subtracted. **c**, cGMP and cAMP dose-response relations at +60 mV, generated with same protocol as in **b**. Averaged data ($\pm$ s.d.) are from 3 patches in each case. Smooth curves are least-squares fits of the Hill equation, $I = C/[C^n + K_{1/2}^n]$, where I is current normalized against that elicited by 1 mM cGMP, and C is cyclic-nucleotide concentration. For cGMP, $K_{1/2} = 20.2\mu\text{M}$, $n = 2.1$; for cAMP, $K_{1/2} = 1,450\mu\text{M}$ and $n = 1.6$. Ratio of maximum cAMP-elicited current to maximum cGMP-elicited current is 0.16. At –60 mV, the activation by cGMP was slightly less effective, with a $K_{1/2}$ about 30% higher (not shown). The inclusion of the phosphodiesterase inhibitor IBMX (0.5 mM) in the bath solution did not change the cGMP dose-response relation, suggesting that any endogenous phosphodiesterase was negligibly active under the experimental conditions or had been washed away. **d**, Inhibition of cGMP-induced current by Ca^{2+} -calmodulin; 20 μM cGMP. Na^+ solution without divalent cations in pipette. Same solution in bath for trace 1, Na^+ solution with 50 μM buffered free Ca^{2+} (see Methods) for trace 2, and with 50 μM

Ca^{2+} plus 250 nM calmodulin for trace 3. Each trace is the average of 5 steps from 0 to +60 mV. Recovery was complete when Ca^{2+} and calmodulin were removed. Three other experiments gave the same results. No effect of calmodulin was seen at 1 mM cGMP, indicating the inhibition involved a change in the apparent cGMP affinity. **e**, The cGMP-activated channel has a low Cl^- permeability. Na^+ solution (100 mM NaCl) without divalent cations was in the pipette. Bath solution was initially the same, then had the NaCl concentration successively changed to the values indicated; for reduced NaCl , sucrose was used as substitute. Top, each trace represents the average of 5 voltage ramps with 1 mM cGMP minus that without cGMP (ramp rate = 120 mV s^{-1}). Bottom, V_{rev} plotted against Na^+ activity in bath. Averaged data from 5 patches (not all NaCl concentrations could be tested on each patch). Straight line has a slope of –58 mV per 10-fold increase in Na^+ activity (Nernstian behaviour). The steeper slope of the experimental relation could have arisen from some junction potential not completely removed by the 1 M KCl agar bridge at the bath electrode, but could not have resulted from any permeability of the channel to Cl^- , which would give a slope gentler than Nernstian. **f**, Measurements of relative permeabilities to basic monovalent cations. Na^+ solution was without divalent cations in pipette. Bath solution had all Na^+ replaced successively by Li^+ , K^+ , Rb^+ and Cs^+ . Small junction potentials at the ground electrode caused by the ion substitutions were ignored. Ramp protocol was similar to that in **e**.

Table 1 Phototransduction mechanisms in various ciliary photoreceptors

Rods and cones	Light-sensitive pinealocytes	Parietal-eye photoreceptors	Scallop hyperpolarizing photoreceptors
Vertebrate	Vertebrate	Vertebrate	Invertebrate
Light ↓ cGMP ↓ cGMP-activated non-selective cation channel Closed ↓ Hyperpolarization	Light ↓ cGMP ↓ cGMP-activated non-selective cation channel Closed ↓ Hyperpolarization	Light ↓ cGMP ↑ cGMP-activated non-selective cation channel Open ↓ Depolarization	Light ↓ cGMP ↑ cGMP-activated K ⁺ -selective channel Open ↓ Hyperpolarization

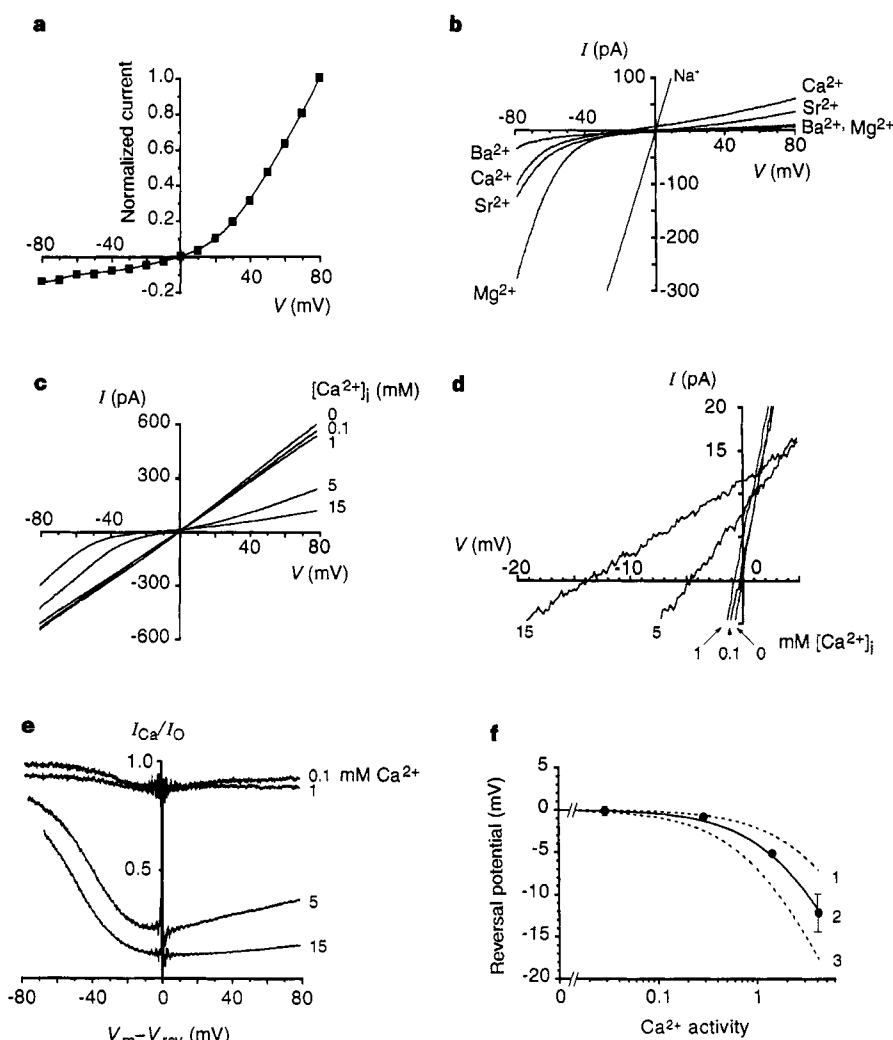


Figure 2 **a**, Current-voltage relation of the cGMP-induced current in the presence of extracellular divalent cations. Averaged data from 3 experiments (s.d. too small to be observable); 1 mM cGMP. The voltage protocol was as in Fig. 1b. Na⁺ solution with 2 mM CaCl₂ and 1 mM MgCl₂ in pipette, and Na⁺ solution without divalent cations in bath. **b**, Divalent cations carry current through the channel. Na⁺ solution without divalent cations in pipette, and iso-osmotic divalent cation solution containing Ca²⁺, Ba²⁺, Sr²⁺ or Mg²⁺ in bath; both were buffered with TMA-HEPES. No outward current was observed if all divalent cations were iso-osmotically replaced by TMA; 1 mM cGMP. The ramp protocol was as in Fig. 1e, f. In the experiment with Ca²⁺, $V_{rev} = -21.7$ mV. For the specific ionic conditions of this experiment, it can be derived that $P_{Ca}/P_{Na} = ([Na^+]_0/4[Ca^{2+}]_i) \times (\exp(-2V_{rev}/\alpha) + \exp(-V_{rev}/\alpha))$, where $\alpha = RT/F$, and $[Na^+]_0$ and $[Ca^{2+}]_i$ are pipette Na⁺ and bath Ca²⁺ activities^{12,29,30}. We thus obtain $P_{Ca}/P_{Na} = 8.1$, broadly

similar to that measured in the following experiment. **c-f**, Measurement of Ca²⁺-permeability. **c**, Influence of different Ca²⁺ concentrations on current-voltage relation. Na⁺ solution without divalent cations in pipette, and Na⁺ solution with indicated free Ca²⁺ concentrations in bath. Solutions were buffered with TMA-HEPES. **d**, Expanded plot of the traces in **c** in the vicinity of the reversal potentials. **e**, Ratio of current with Ca²⁺ to that without Ca²⁺, plotted against $V_m - V_{rev}$. Data were calculated from **c**. **f**, Reversal potential plotted against bath Ca²⁺ activity. Averaged data from 4 experiments. Curves are drawn according to the equation $V_{rev} = -(\alpha/2) \ln(4[Ca^{2+}]/([Na^+])(P_{Ca}/P_{Na}) + 1)$, where $\alpha = RT/F$ and $[Na^+]$, $[Ca^{2+}]$ are the symmetrical Na⁺ and bath Ca²⁺ activities, respectively. This equation can be derived based on the ionic conditions here^{12,29,30}. The curves fits are with $P_{Ca}/P_{Na} = 5.0$ (curve 1), 10.3 (2) and 20.0 (3).

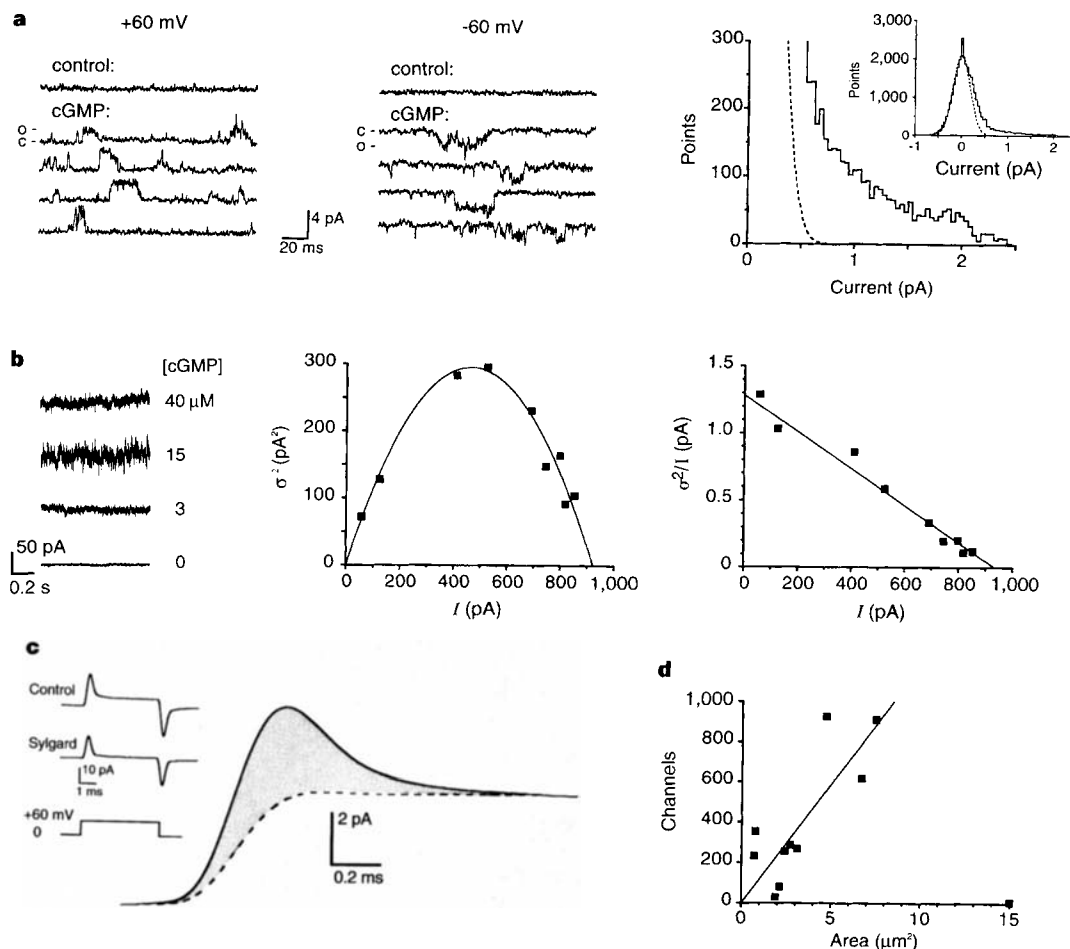


Figure 3 Measurement of channel density on membrane. In these experiments, symmetrical Na^+ solutions without divalent cations were used, with 2-kHz low-pass filtering (8-pole Bessel) and 5-kHz digitization. **a**, Single-channel recordings; data are all from the same patch. Sample traces were from 300-ms voltage steps from 0 to ± 60 mV at $0.6 \mu\text{M}$ cGMP. The labels 'c' and 'o' indicate closed and open states of the channel, respectively. The amplitude histogram on the right is for +60 mV, derived from all digitized points in ~ 6 s of data. **b**, Variance analysis to derive the effective single-channel current (i), the open probability (P_o), and the number of channels (N). Left, sample records of macroscopic currents induced by different cGMP concentrations to show the associated noise; +60 mV. Middle, variance (σ^2) of the current plotted against the mean current (I). cGMP concentrations were 3, 6, 10, 15, 20, 25, 30, 40 and $1,000 \mu\text{M}$, respectively. The current variance at each cGMP concentration was calculated from 6.5 s of continuous recordings. The variance recorded in the absence of cGMP was already subtracted; same patch as on the left. Right, σ^2/I plotted against I for the same data. The curve fits in both panels are from $\sigma^2 = Ii - I^2/N$, with $i = 1.28$ pA and $N = 721$. The maximum current, induced by 1 mM cGMP, is 850 pA, and the I value extrapolated to zero variance is 923 pA; thus P_o is 0.921. **c**, Measurement of patch area. The strategy consisted in eliminating the patch capacitance by gently pressing the patch-pipette tip with an attached excised patch against a surface of

Sylgard elastomer, and measuring the resulting decrease in the transient capacitive current associated with a voltage jump^{15,16}. Inset, capacitive current transients associated with a brief voltage step from 0 to +60 mV under two conditions: with the membrane patch at the pipette tip within a few micrometres from ('control'), and just touching ('Sylgard'), a small slab of Sylgard, respectively; 0 cGMP. Symmetrical Na^+ solutions without divalent cations. Each trace is the average of 15 trials. The difference between the two traces for the 0 to +60 mV jump is shown on the right. The dashed trace represents a scaling of the instrumentation step response, obtained by connecting a resistor (values of 1–100 M Ω gave similar results) across the inputs on the headstage of the patch-clamp amplifier (Axopatch-1D; Axon Instruments). The membrane-patch capacitive charge displacement (shaded area) associated with the 60 mV jump is 1.86×10^{-15} C, giving a patch capacitance of 3.1×10^{-14} F. Assuming a specific capacitance of $1 \mu\text{F cm}^{-2}$ for the cell membrane, we obtain a patch area of $3.1 \mu\text{m}^2$. The maximum current, I_{max} , induced by cGMP (1 mM) in this patch was 349 pA. From $N = I_{\text{max}}/iP_o$, with $i = 1.37$ pA and $P_o = 0.947$ (see text), we obtain $N = 269$, giving a channel density of $87 \mu\text{m}^{-2}$. **d**, Relation between number of channels and patch area. Collected results from 11 patches; +60 mV. The straight line is a linear-regression fit to the left 10 data points, with a slope of 118 channels μm^{-2} . The arithmetic average from all 11 patches was 143 channels μm^{-2} .

absence of divalent cations seemed to be 1–2 pA, but the rapid flickers made an objective value assignment difficult (Fig. 3a). Instead, we analysed the variance, σ^2 , of the macroscopic current, I , at +60 mV and at different cGMP concentrations using the relation $\sigma^2 = Ii - I^2/N$ (ref. 14). From four experiments (see example in Fig. 3b), we obtained i (the effective single-channel current) as 1.37 ± 0.20 pA, and P_o as 0.947 ± 0.024 (mean $\pm$ s.d.). Using these values and $N = I_{\text{max}}/iP_o$, N was evaluated for 11 patches with I_{max} measured at +60 mV. The area of each patch was estimated from capacitive current transients^{15,16} (Fig. 3c). There was an approximately linear relation between channel number and patch area (Fig. 3d), giving a fairly uniform channel density of ~ 143

channels per μm^2 (Fig. 3 legend), remarkably similar to that measured from the amphibian rod outer segment^{16,17}. By comparison, 7 out of 13 membrane patches excised from the adjacent inner segment responded to cGMP, but showed only 1–6 channels, as counted from the discrete levels in the current recordings. The areas of 3 of these patches were measured, giving a mean density of 1.37 channels per μm^2 . Thus the cGMP-gated channel seems to be specifically concentrated on the outer segment. Channel activity other than that activated by cGMP was not observed from outer segment patches, but was observed from inner segment patches. One phototransduction pathway proposed for the *Limulus* photoreceptor is that light-activated production of

inositol-1,4,5-trisphosphate (InsP₃) increases intracellular Ca²⁺, which somehow elevates cGMP⁹. Such a mechanism is unlikely to mediate phototransduction in the parietal-eye photoreceptor because the structure of its outer segment⁵ precludes the possibility of any intracellular compartment for storing Ca²⁺, to be released by InsP₃. Furthermore, bath application of 200 μ M InsP₃ had no effect on the inside-out patches excised from the outer segment (data not shown), indicating the absence of InsP₃ receptor channels on the plasma membrane as well. Therefore, it appears that the cGMP-gated channel is coupled not to an InsP₃ pathway, but to a more conventional cGMP signalling cascade, to mediate phototransduction. Recordings from intact parietal-eye photoreceptors are also consistent with this idea (W.-H. Xiong and K.-W.Y., unpublished data). The depolarizing light response and the non-selectivity of the cGMP-gated cation channel together suggest that light increases cGMP in the cell, which is the opposite to the situation in rods and cones.

Like the pineal gland, the parietal eye is developmentally an outgrowth of the diencephalon⁵. In lower vertebrates and birds, the pineal gland is light-sensitive^{18–21}. The pineal photoreceptor hyperpolarizes to light owing to a membrane-conductance decrease as in rods and cones^{18,19,21}, and it also has a cGMP-activated, non-selective cation channel^{22,31}. By contrast, the parietal eye photoreceptor depolarizes to light under dark-adapted conditions, a feature that is characteristic of most known invertebrate photoreceptors with a rhabdomeric (microvilli-derived) photosensitive structure²³. Indeed, it is the only known ciliary photoreceptor, vertebrate or invertebrate, that depolarizes to light. The phototransduction mechanism in rhabdomeric photoreceptors is still unclear, but existing evidence argues strongly for the involvement of a phosphoinositide pathway^{7–9}. Our findings, then, together with work on a hyperpolarizing photoreceptor in scallop²⁴, suggest a fundamental principle: ciliary photoreceptors, whether hyperpolarizing or depolarizing, vertebrate or invertebrate, uniformly use a cGMP signalling cascade, without involving InsP₃, for phototransduction. Thus the polarity of the light response does not speak for the underlying phototransduction mechanism. The ciliary photoreceptors with known transduction pathways are summarized in Table 1. Despite the common cGMP-signalling motif, the details of phototransduction do vary among these cell types. In rods and cones, and apparently light-sensitive pinealocytes, light lowers cGMP to close a cGMP-activated, non-selective cation channel, producing a hyperpolarization. In the hyperpolarizing photoreceptors of scallop, the light response results from the opening of a cGMP-activated K⁺ channel, presumably as a result of rise in cGMP²⁴. Finally, as mentioned above, light also appears to elevate cGMP in the photoreceptors of the parietal eye, but in this case they produce a depolarization through the opening of a cGMP-activated, non-selective cation channel. For completeness, it is worth mentioning an extra-ocular neuron in the abdominal ganglion of the mollusc *Onchidium verruculatum* that depolarizes to light, apparently as a result of the closure of a cGMP-activated K⁺ channel²⁵. Because it lacks any obvious photosensitive structure (T. Gotow, personal communication), however, this neuron cannot be included in our generalization. Further understanding of the phototransduction processes in various ciliary photoreceptors may provide insights into the evolutionary lineage of these cells. The rich variations on a common motif found among ciliary photoreceptors raise the question of whether the phototransduction mechanisms in different rhabdomeric photoreceptors (the other major type in the animal kingdom), such as those in *Limulus*, *Drosophila* and squid, also show variations on a shared design.

Note added in proof: A related generalization about ciliary photoreceptors has recently been proposed²⁶. □

Methods

Preparation. The parietal eye, still attached to the overlying skin, was dissected free from the skull and immersed in CO₂-independent medium (CIM; Gibco)

containing collagenase II (2 mg ml⁻¹; Sigma) for 12 min. After washes in CIM, the eye was removed from the overlying skin with fine forceps. The eye was then placed in CIM containing pronase (2 mg ml⁻¹; Boehringer) for 12 min, after which the eye capsule could be removed. Finally, the neural tissue was treated with 1 mg ml⁻¹ trypsin (Sigma) in CIM for 15 min, rinsed in CIM for 30 min, and triturated gently with glass pipettes of progressively smaller diameters (400–100 μ m). The cells were plated on glass coverslips pretreated with concanavalin A, and used within 4–6 h. The cell dissociation and subsequent patch-clamp recordings were all done under room light and at 23–25 °C.

Electrophysiology. The patch pipettes were made from thick-walled borosilicate glass capillaries and had tip lumen diameters of ≤ 1 μ m. In single-channel recordings, as well as noise and capacitive current measurements, the exterior of the pipette tip was coated with Sylgard to reduce electrical noise and wall capacitance. Sodium ion solution without divalent cations generally contained (in mM) 140 NaCl, 0.5 sodium EGTA, 0.5 sodium EDTA, 10 sodium-HEPES, pH 7.6. In the experiments of Figs 1, 2a and 3, the sodium ion solution also contained 5 mM KCl; in the experiment of Fig. 1e, 100 mM NaCl was used instead. Solutions containing buffered free Ca²⁺ had, instead of sodium EGTA and sodium EDTA, 2 mM sodium-NTA (nitrilotriacetic acid) and the following added CaCl₂ concentrations: 920 μ M (50 μ M free Ca²⁺) and 1.312 mM (100 μ M free Ca²⁺). For higher divalent-cation concentrations, no buffer was added. In the experiments of Fig. 2b–f, tetramethylammonium (TMA)-HEPES instead of sodium-HEPES was used, and the iso-osmotic divalent-cation solutions in Fig. 2b contained 93 mM CaCl₂, BaCl₂, SrCl₂ or MgCl₂ instead of the NaCl. Finally, in the experiments of Figs 1e and 2c–f, an agar bridge containing 1 M KCl was added between the Ag–AgCl ground electrode and the bath solution to minimize the junction potential. Unless otherwise specified, the concentration of cGMP in the bath solution was 1 mM. The sodium salt of cGMP was used throughout, except for the experiment in Fig. 2b, where the free acid of cGMP was used instead. In all calculations involving reversal potentials, ion activities^{27,28} instead of concentrations were used; the assumption $\gamma_{2+} = (\gamma_{+})^2$ (ref. 28) was used in calculating the Ca²⁺ activity. In all figures, outward membrane current (flowing from the bath to the pipette side) has a positive sign.

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Insensitivity to anaesthetic agents conferred by a class of GABA_A receptor subunit

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A common feature of general anaesthetic agents is their ability to potentiate neuronal inhibition through GABA_A (γ-aminobutyric acid) receptors¹. At concentrations relevant to clinical anaesthesia, these agents cause a dramatic stimulation of the chloride currents that are evoked by the binding of the natural ligand, GABA. Although there is widespread evidence that the sensitivity of GABA_A receptors to anaesthetic agents is heterogeneous²⁻⁴, the structural basis of these differences is largely unknown. Variations in subunit composition can have profound effects on the sensitivity of GABA_A receptors to modulatory agents such as benzodiazepines⁵. However, strict subunit specificity has not been demonstrated for the potentiating effects of anaesthetic agents⁶⁻¹⁰. Here we describe a new class of human GABA_A receptor subunit (ε) that can assemble with α- and β-subunits and confer an insensitivity to the potentiating effects of intravenous anaesthetic agents. The ε-subunit also abolishes the normal outward rectification of recombinant receptors in which it assembles. The expression pattern of this subunit in the brain suggests a new target for manipulation of neuronal pathways within the basal ganglia.

In mammals, the 13 known subtypes of GABA_A receptor subunits have been categorized within four structural classes (α1-6, β1-3, γ1-3 and δ). These subunits are thought to assemble in different pentameric complexes, with most functional receptors containing α/β/γ or α/β/δ subunit combinations¹¹. An expressed sequence tag (EST) that encodes a new class of subunit was identified by searching an EST database with a peptide consensus sequence of known family members. Using the sequence of this EST, flanking cDNA sequences were amplified from a hippocampal cDNA library by anchored polymerase chain reaction (PCR). The longest con-

tiguous cDNA that was identified by this method (3.1 kilobases), contains a large open reading frame that encodes a polypeptide of 506 amino-acid residues (Fig. 1). This amino-acid sequence is most closely related to GABA_A receptor γ-subunits (38-43% amino-acid identity) and α-subunits (28-30%); it displays less similarity to other GABA and glycine receptor subunits (<25%). The maximum levels of sequence identity are similar to those found between the different classes of GABA_A receptor subunits¹¹, and justified its categorization within a distinct subunit class (ε).

Expression of ε-subunit mRNA in various tissues was examined by northern analysis. Using probes that were derived from either the 3'- or 5'-ends of the ε-subunit cDNA, two species of RNA (2.8 and 3.2 kb) were readily detectable in several brain regions (Fig. 2). Although these transcripts were barely detectable in samples of whole brain, they were relatively enriched in amygdala and thalamus, and were particularly abundant in the subthalamic nucleus. The subthalamic nucleus is known to receive major GABA-mediated inputs, with particularly dense projections arising from the globus pallidus¹². Notably, the relatively low levels of γ- and δ-subunit transcripts in rat subthalamic nucleus¹³ are consistent with expression of alternative receptor subunits in this tissue.

The biophysical and pharmacological properties that are conferred to GABA_A receptors by the ε-subunit were examined after transient expression in HEK-293 cells. Cells that were transfected with only the ε-subunit cDNA did not express binding sites of the GABA_A receptor ligands [³H]muscimol or [³⁵S]t-butyl bicyclophosphorothionate (TBPS). These cells also failed to exhibit chloride currents in response to GABA or glycine (each 100 μM). The ε-subunit is therefore unlike the p class of receptor subunits¹¹ or the α class of glycine-receptor subunits¹⁴, which can each assemble homomeric chloride channels that are gated by these ligands. When cells were co-transfected with cDNAs encoding the ε-subunit, and either an α-subunit (α1, α2 or α6) or a β-subunit (β1 or β3), there was also a failure to detect expression of any ligand-binding activities. Therefore the ε-subunit does not behave like a typical GABA_A receptor α- or β-subunit¹¹. As expected, transfection of cells with a combination of α-, β- and ε-subunits resulted in the expression of GABA-activated currents (Fig. 3) and [³⁵S]TBPS binding sites (Fig. 4). However, the transfected cell membranes did not bind either [³H]flunitrazepam or [³H]flumazenil. Therefore the ε-subunit is also unlike the γ-class of subunits, which confers a sensitivity to benzodiazepines when expressed with α- and β-subunits¹¹. The remaining subunit class, δ, has no unique pharmacological features to compare, but can be distinguished from the ε-subunit by its sensitivity to barbiturates (ref. 15, and see below).

ε subunit	MLSKVLPVLLGILLILQSRVEGPQTESKNEASSRDVVYGPQPQLNQLLSEETKSTETETGSRVKGKLEASRIL	75
Consensus	L Y RP G S M Y Q W D RL L L	
ε subunit	WIPDTFFRNKRTHHEITMPNQMVRIYKDGKVLTYTIRMTIDAGCSLHMLRFPMDSHSCPLSFSSFSYPENEMII	225
Consensus	W PDT N H T N R G LY R T C L P D C L SY Y	
ε subunit	KWENFKLEINEKNSWKLFQDFDTGVSNKTEIITTPVGDFMVMITFFNVSRFRGYVAFQNYVPSSVTIMLSWVSWF	300
Consensus	W L Q G F L R G Q Y P M1 S VSEW	
ε subunit	IKTESAPARTSLGITSVLITMTLGTFSRKNFPRVSYYITALDFYIAICFVFCFALLEFAVLNFLIYNQTKAHASP	375
Consensus	AR G TTVLIMTF R LP A D C FVF AL E M2 M3	
ε subunit	KLRHPRINSRAHARTRARSACARQHQAFCVQIVITTEGSDGEERPSCSAQQPPSPGSGPEGPRSLCSKLACCEWC	450
Consensus	KRFKKYFCMVPDCEGSTWQQGRLCIHVYRLDNYSRVFPVTFVFFNVLYWLVCNL	506
	D R FP F N YW Y M4	

Figure 1 Amino-acid sequence of the human ε-subunit. The ε-subunit polypeptide is aligned with a consensus sequence of all known mammalian GABA_A receptor subunits. The potential signal sequence (S) and four putative transmem-

brane domains (M1-M4) are highlighted by lines under the corresponding peptide sequences. Eight differences between the ε-subunit sequence and the consensus are indicated by diamonds.

There was little difference between the concentration–response relationships for GABA-activated chloride currents recorded from cells transfected with $\alpha 2/\beta 1$ or $\alpha 2/\beta 1/\epsilon$ cDNAs (Fig. 3a). As expected, the selective GABA_A receptor inhibitors picrotoxin (10 μ M) and bicuculline (10 μ M) blocked currents mediated by the $\alpha 2/\beta 1/\epsilon$ subunit combination by $82.5 \pm 7.4\%$ ($N = 3$) and $73.6 \pm 9.3\%$ ($N = 4$), respectively. However, a comparison of current–voltage relationships demonstrated that the ϵ -subunit can assemble with α - and β -subunits and confer new biophysical properties. Previous studies have demonstrated that native and recombinant GABA_A receptors exhibit a pronounced outward rectification^{16–19}. Here this was confirmed with cells expressing $\alpha 2/\beta 1$ or $\alpha 1/\beta 3$ subunits, which displayed larger GABA-evoked currents when clamped at positive potentials than at the corresponding negative values (Fig. 3b, c). In marked contrast, inclusion of the ϵ -subunit with either subunit combination yielded currents that exhibit a linear relationship to voltage (Fig. 3b). The cause of outward rectification at GABA_A receptors is controversial^{18–21}, and any of the proposed mechanisms could be influenced by inclusion of the ϵ -subunit. However, it is notable that the ϵ -subunit displays an unusually high density of basic residues close to the M3 segment (residues 376–401) which may affect rectification properties by sequestering chloride ions near the intracellular face of the ion channel.

Having demonstrated that the ϵ -subunit can assemble with other GABA_A receptor subunits, its effects on receptor pharmacology were examined. Unexpectedly, the presence of the ϵ -subunit was found to have a pronounced effect on the sensitivity of GABA_A receptors to intravenous anaesthetic agents. This was demonstrated by both radioligand binding and by measurement of GABA-evoked currents (Fig. 4). First, in common with studies on other α/β subunit combinations^{17,22}, the anaesthetic steroid, 5 α -pregnan-3 α -ol-20-one, was found to have a biphasic effect on [³⁵S]TBPS binding to cells expressing $\alpha 2/\beta 1$ subunits. In contrast, there was no stimulatory phase to the dose–response curve when using the $\alpha 2/\beta 1/\epsilon$ subunit combination (Fig. 4a). The functional correlate of this assay examined the effect of pregnanolone (10 nM to 1 μ M) on submaximal GABA-activated currents recorded from

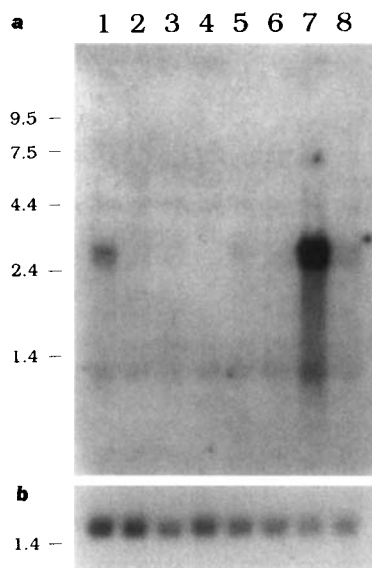


Figure 2 Distribution of ϵ -subunit mRNA in human brain regions. **a**, Hybridization of poly(A)⁺ RNA from amygdala (lane 1), caudate nucleus (2), corpus callosum (3), hippocampus (4), whole brain (5), substantia nigra (6), subthalamic nucleus (7) and thalamus (8) with a ³²P-labelled fragment of the 3'-untranslated ϵ -subunit cDNA. **b**, The same blot was reprobed with a ³²P-labelled fragment of the human GAPD cDNA.

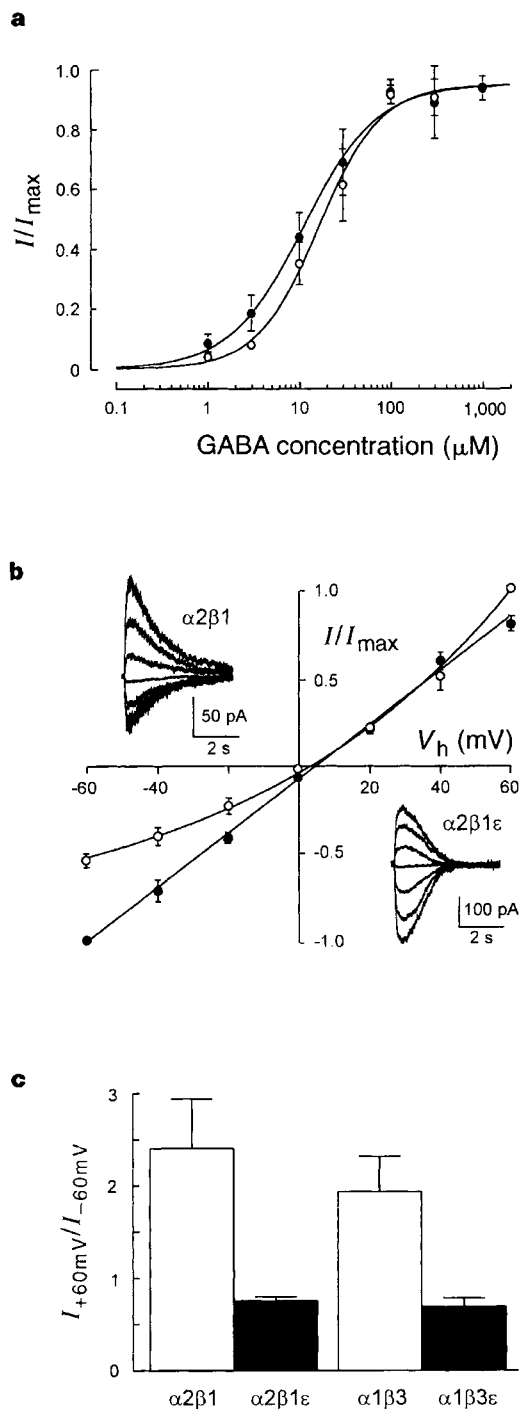


Figure 3 Effects of the ϵ -subunit on activation of receptors by GABA. **a**, Concentration-dependent activation of currents recorded from cells transfected with either $\alpha 2/\beta 1$ (open circles) or $\alpha 2/\beta 1/\epsilon$ (filled circles) cDNAs. From the logistic fit³⁰, the EC₅₀ values are 16.4 and 11.2 μ M, and Hill slopes are 1.3 and 1.1, respectively. Data points represent current amplitudes recorded from at least 3 cells, normalized to the maximum current of each cell. **b**, Current–voltage relationships for responses evoked by 100 μ M GABA, recorded from cells transfected with $\alpha 2/\beta 1$ (open circles) or $\alpha 2/\beta 1/\epsilon$ (filled circles) cDNAs. From the respective exponential and linear fits to the data points³⁰, currents reversed at 4.4 and 5.7 mV. Data points represent the mean current amplitudes recorded from at least 4 different cells and normalized to the peak current amplitude of each cell. The insets are superimposed traces showing averages of two leak-subtracted currents recorded from individual cells when held at voltages between 60 and –60 mV. **c**, The ratio of GABA (100 μ M)-evoked current amplitudes, recorded at 60 and –60 mV, for different α/β and $\alpha/\beta/\epsilon$ subunit combinations. Data are mean values from 4–18 cells.

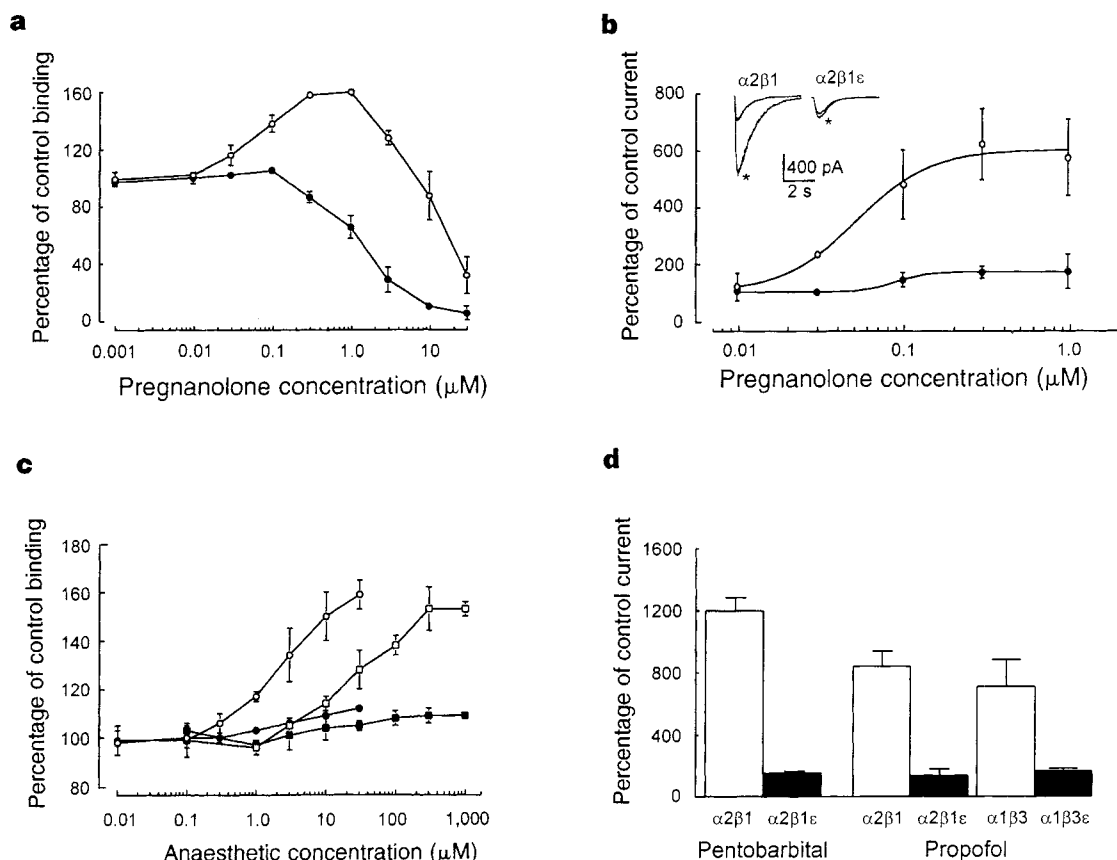


Figure 4 Effects of the ϵ subunit on the modulation of GABA_A receptors by anaesthetic agents. **a**, Modulation by pregnanolone of [³⁵S]TBPS binding to the membranes of cells transfected with either $\alpha 2/\beta 1$ (open circles) or $\alpha 2/\beta 1/\epsilon$ (filled circles) cDNAs. **b**, Modulation of GABA (100 μ M)-evoked currents by pregnanolone, recorded from cells expressing $\alpha 2/\beta 1$ (open circles) or $\alpha 2/\beta 1/\epsilon$ (filled circles) cDNAs. Data points represent mean values from at least 4 cells, fitted using the logistic function³⁰. The insets are superimposed traces showing

currents recorded in the absence or presence (asterisks) of pregnanolone (100 nM). **c**, Modulation of [³H]muscimol binding to cell membranes by pregnanolone (circles) and pentobarbital (squares) after transfection with either $\alpha 1/\beta 3$ (open symbols) or $\alpha 1/\beta 3/\epsilon$ (filled symbols) cDNAs. **d**, Effects of pentobarbital (100 μ M) and propofol (3 μ M) on GABA (100 μ M) evoked currents recorded from cells expressing different α/β and $\alpha/\beta/\epsilon$ subunit combinations. Histogram bars represent mean data from at least 4 cells.

cells expressing $\alpha 2/\beta 1$ or $\alpha 2/\beta 1/\epsilon$ -subunit combinations (Fig. 4b). In the absence of the ϵ -subunit, currents were potentiated by up to 521%, with a half-maximal effective concentration (EC_{50}) value of 53 nM. In contrast, currents recorded from the $\alpha 2/\beta 1/\epsilon$ subunit combination were relatively insensitive to the steroid. No significant effects were observed at concentrations up to 100 nM pregnanolone, and only minor effects (up to 73% potentiation) were detected at high concentrations of the steroid (Fig. 4b).

This effect of the ϵ -subunit was examined further using different subunit combinations and additional anaesthetic agents. The ability of anaesthetic agents to stimulate [³H]muscimol binding to cell membranes is a reliable indicator of their ability to stimulate GABA-evoked currents recorded from intact cells²³. After transfection with $\alpha 1/\beta 3$ subunits, [³H]muscimol binding to cell membranes was stimulated by up to 60% in the presence of pregnanolone or the anaesthetic agent pentobarbital. However, when the ϵ -subunit was expressed with this combination, the maximal stimulation dropped to less than 15% (Fig. 4c). The dose-response curves for a third anaesthetic agent, propofol (2,6-diisopropylphenol), were similar to those of pentobarbital (M.C.H., unpublished results). Consistent with the effect on ligand binding, inclusion of the ϵ -subunit with different α/β subunit combinations blocked the ability of pentobarbital (100 μ M) and propofol (3 μ M) to enhance GABA-evoked currents (Fig. 4d).

High concentrations of anaesthetic agents can activate GABA_A receptors in the absence of GABA¹¹. Notably, cells expressing $\alpha/\beta/\epsilon$

subunit combinations retained their sensitivity to this direct activation. Pregnanolone (10 μ M), propofol (100 μ M) and pentobarbital (1 mM) each evoked picrotoxin-sensitive currents recorded from cells expressing $\alpha 2/\beta 1/\epsilon$ or $\alpha 1/\beta 3/\epsilon$ cDNAs (0.2–2.1 nA, $N = 4$ –6). The loss of sensitivity to the potentiating effects, but not the activating effects, of intravenous anaesthetic agents supports the view that the two responses are mediated by different anaesthetic binding sites on the receptor complex⁹. The δ subunit has been reported to confer analogous properties with respect to anaesthetic steroids²⁴ but not barbiturates¹⁵. Future use of ϵ - and δ -subunit fragments for chimaeric subunit construction may provide insights to the structural basis of different anaesthetic actions at GABA_A receptors.

It remains possible that the ϵ -subunit can confer additional pharmacological properties to GABA_A receptors that have yet to be uncovered. In view of its abundant expression in the subthalamic nucleus, identification of these properties could have important implications for the treatment of movement disorders, including Parkinson's disease^{25,26}. Its restricted expression pattern in the human brain provides an opportunity for relatively selective manipulation of this important nucleus of the basal ganglia. □

Methods

Isolation of the ϵ -subunit cDNA. The database dbEST (<http://www.ncbi.nlm.nih.gov/dbEST/index.html>) was searched using the TBLASTN algorithm. The query sequence was a consensus polypeptide containing amino-acid

residues that are invariant between all known mammalian GABA_A receptor subunits (Fig. 1). Oligonucleotide primers were designed from an EST sequence (GenBank accession no. R07883) to amplify 5' and 3' flanking sequences from cDNA libraries²⁷ by anchored PCR. Amplification at 95 °C for 45 s, 60 °C for 60 s and 72 °C for 2 min was performed for 35 cycles using the XL-PCR system (Perkin Elmer). Reaction products were purified from agarose gels and sequenced directly. The ϵ -subunit open reading frame was amplified from a hippocampal cDNA library as described above using primers containing nucleotides 11–34 (sense) and 1,569–1,592 (antisense) of the ϵ -subunit cDNA sequence (GenBank accession no. U66661). The reaction products were ligated into pCDM8 (Invitrogen), and clones were sequenced over their entire lengths to ensure that no mutations had been introduced.

Northern blot analysis. Samples of approximately 2 μ g poly(A)⁺ RNA (Clontech) were electrophoresed on a 1.2% formaldehyde agarose gel, transferred to a nylon membrane and hybridized with a ³²P-labelled fragment of the 3'-untranslated ϵ -subunit cDNA (nucleotides 1,939–2,945). The blot was washed at 60 °C in 0.1 $\times$ SSC, 0.1% SDS before exposure. The blot was then stripped of probe by boiling in 0.5% SDS, and rehybridized with a ³²P-labelled fragment from the 5' end of the ϵ -subunit cDNA (nucleotides 19–347). Finally, the same blot was stripped and reprobbed with a ³²P-labelled fragment of the human GAPD cDNA²⁸ (nucleotides 789–1,140).

Transient transfection of subunit cDNAs. Transfection of human embryonic kidney cells (HEK-293) with subunit cDNAs was performed with a calcium phosphate-DNA precipitate in HEPES buffer. After incubation with the precipitate for 24 h, the cells were washed and cultured for a further 48–72 h before use. The subunit cDNAs that were used for transfection were: human α 2 and β 1, cloned in pCIS2; rat α 1, rat α 6, rat β 1, human β 3 and human ϵ , cloned in pCDM8.

Radioligand binding assays. Transfected cells were washed twice with phosphate-buffered saline and collected by scraping into an ice-cold solution containing (in mM) Tris-HCl 10, EDTA 1, NaCl 100, pH 7.5 (TEN). The cells were collected by centrifugation (5,000g, 10 min), hand homogenized in TEN, and a crude membrane fraction was obtained by centrifugation (30,000g, 30 min). The membrane pellet was washed twice with TEN, and resuspended in TEN at a protein concentration of ~3 mg ml⁻¹. The binding of [³⁵S]TBPS (20 nM; 88.7 Ci mmol⁻¹; NEN) to membranes (50–100 ng protein) was assayed by filtration after incubation for 120 min at 25 °C in 100 μ l of a solution containing Tris-HCl (20 mM), NaCl (1 M), pH 7.5. Nonspecific binding was determined in the presence of picrotoxin (100 μ M), and was equal to the binding of mock-transfected cell membranes (<0.03 pmol per mg protein). The binding of [³H]muscimol (30 nM; 19.5 Ci mmol⁻¹; NEN) was assayed after incubation of membranes for 60 min at 0 °C in 100 μ l of TEN. Nonspecific binding was determined in the presence of GABA (1 mM). The binding of [³H]flunitrazepam and [³H]flumazenil (each 1–50 nM) was assayed essentially as described previously²⁹. All binding data are mean $\pm$ s.d. of three independent transfections.

Electrophysiology. During whole-cell recordings, cells were superfused (5 ml min⁻¹) with a solution of (in mM): NaCl 140, KCl 4.7, MgCl₂ 1.2, CaCl₂ 2.5, glucose 11, and HEPES 10, pH 7.4. The electrode solution contained KCl 140, MgCl₂ 2.0, EGTA 11, and HEPES 10, pH 7.4. The electrode solution was supplemented with 100 μ M ATP to prevent current rundown in recordings from cells expressing α 2/ β 1 and α 2/ β 1/ ϵ . When examining their agonist action, GABA, glycine and anaesthetics were applied locally, by pressure (70 kPa) ejection from micropipettes situated 50 μ m from the cell. For modulation experiments, in which anaesthetics, bicuculline methiodide and picrotoxin were bath applied, GABA (100 μ M) was ejected for brief durations (5–30 ms) that activated approximately 10% of maximum current amplitude. For concentration-response experiments, GABA was applied for 1 s from low-resistance pipettes³⁰. Currents were filtered (1 kHz) and recorded at -60 mV (unless otherwise stated) using an Axopatch 200A amplifier. Data were simultaneously digitized for storage on VCR tapes and the hard drive of an IBM PC. Currents were analysed and reproduced using pCLAMP software (Axon). All electrophysiological data are mean $\pm$ s.e.m.

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Mechanism of resistance of African trypanosomes to cytotoxic human HDL

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Trypanosoma brucei brucei, the causative agent of ngana in cattle, is non-infectious to humans because of its sensitivity to the cytolytic activity of normal human serum¹. The toxin in normal human serum is human haptoglobin-related protein (Hpr)^{2–5} which is found either as an apolipoprotein associated with a minor subclass of high-density lipoprotein (HDL), named trypanosome lytic factor (TLF1)^{6–8}, or as an unstable, high-molecular-mass protein complex known as TLF2 (refs 5, 9–12). TLF-mediated lysis of *T. b. brucei* requires binding, internalization and lysosomal targeting¹³. The human sleeping-sickness trypanosome, *Trypanosoma brucei rhodesiense* is resistant to TLF. Our studies reveal that resistant trypanosomes fail to endocytose TLF yet continue to bind TLF through cell-surface receptors. On the basis of these results, we conclude that one mechanism of resistance of human sleeping-sickness trypanosomes to human serum is decreased internalization of receptor-bound TLF.

The human and animal diseases caused by subspecies of African trypanosomes are biochemically and morphologically indistinguishable¹⁴. Resistance to normal human serum is an unstable phenotype in *T. b. rhodesiense* because trypanosomes susceptible to lysis by human serum rapidly accumulate upon serial passage in laboratory rodents^{15,16}. The mechanism of resistance to normal human serum is poorly understood^{17–19}. Reports linking changes in surface antigen expression to loss of serum resistance in *T. b. rhodesiense* prompted us to investigate the role of the variant surface glycoprotein (VSG) in the TLF-mediated

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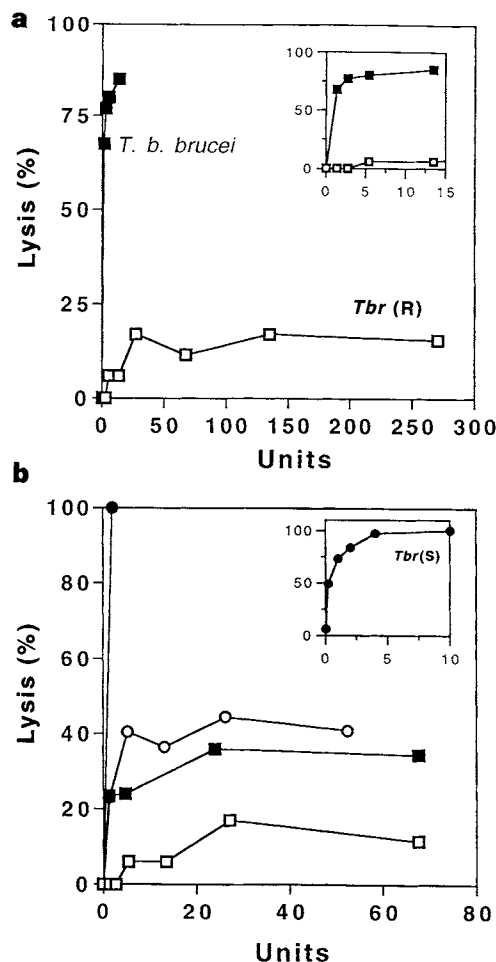


Figure 1 TLf-mediated lysis in *T. b. brucei*, and *T. b. rhodesiense*. **a**, Lysis of *T. b. brucei* (black squares) and *T. b. rhodesiense*, which is resistant (white squares). **b**, Serial passage of the *Tbr (R)* clone. *T. b. rhodesiense* TLf-resistant clone, *Tbr (R)* (white squares), *Tbr (R)* following 16 three-day passages in mice (black squares), *Tbr (R)* following 17 three-day passages in mice (white circles), clone derived from passage 17 *Tbr (R)* which is sensitive to TLf (*Tbr (S)*, black circles). Insets, lysis of *T. b. brucei* (**a**) and *Tbr (S)* (**b**) over the full range of TLf concentrations.

lysis^{19–21}. In order to investigate the influence of the VSG in susceptibility to human serum lysis we isolated *T. b. rhodesiense* clones differing in their susceptibility to lysis. Clonal populations were prepared from a human serum resistant *T. b. rhodesiense* isolate (Fig. 1a). This clonal population, designated *Tbr (R)*, was not lysed by human serum or purified TLf at concentrations more than 200-fold higher than those needed to lyse serum-sensitive *T. b. brucei* stocks (Fig. 1a, and data not shown). When *Tbr (R)* was passed at 2–3-day intervals in irradiated mice, a portion of the population became serum-sensitive. After 17 mouse passages, about 45% of the trypanosomes were susceptible to TLf (Fig. 1b). Cell clones were prepared from this population and trypanosomes highly susceptible to TLf were identified and designated *Tbr (S)* (Fig. 1b). VSG was purified from the *Tbr (R)* and *Tbr (S)* clonal cell lines²². The VSG from *Tbr (R)* and *Tbr (S)* specifically recognized the 63K VSG in the *T. b. rhodesiense* populations but did not react with the VSG from an unrelated *T. b. brucei* (ILTat 1.3) cell extract on western blots (data not shown). Monoclonal antibodies raised against *Tbr (R)* and *Tbr (S)* VSG revealed similar cross-reactivity by western blot analysis (data not shown). In addition, antisera raised against VSG from both *Tbr (R)* and *Tbr (S)* are capable of *in vitro* antibody-mediated killing both *T. b. rhodesiense* populations, but not trypanosomes

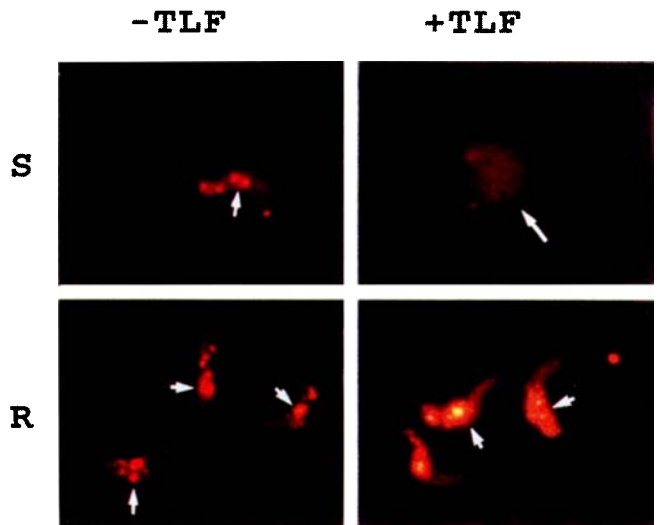


Figure 2 Fluid-phase endocytosis of dextran by the *Tbr (R)* and *Tbr (S)* clones. Cells were preincubated with rhodamine-labelled dextran $\pm$ 4 units of TLf. In the absence of TLf, both *Tbr (R)* and *Tbr (S)* cells accumulate dextran in perinuclear regions of the cells (arrows). In the presence of TLf, punctate dextran fluorescence is visible in the *Tbr (R)* cells but only a diffuse overall fluorescence is seen in the *Tbr (S)* cells, indicating lysosome disruption by TLf.

expressing a different VSG (data not shown). Finally, purified VSG from *Tbr (R)* and *Tbr (S)* were identical in amino-acid composition (data not shown). Taken together, our results show that these TLf-resistant and -susceptible cell lines express the same VSG.

As resistance of *T. b. rhodesiense* to TLf is not a direct result of VSG composition, we decided to evaluate whether the mechanism of resistance was similar to the previously established mechanism of TLf resistance found in the procyclic insect form of *T. b. brucei*. Procyclics are resistant to the toxic effects of TLf because of their reduced ability to take up macromolecules²³. To test whether fluid-phase endocytosis was reduced in the *Tbr (R)* population, we incubated bloodstream trypanosomes with fluorescently labelled dextran at 37 °C and monitored uptake by fluorescence microscopy (Fig. 2). Fluorescence was localized to the perinuclear region of both *Tbr (R)* and *Tbr (S)* cells, consistent with its accumulation in lysosomes¹³. Upon exposure to TLf, the punctate perinuclear fluorescence was maintained in *Tbr (R)* cells, but was replaced in *Tbr (S)* cells by a diffuse cytoplasmic fluorescence (Fig. 2). This is consistent with lysosomal membrane disruption, as observed in TLf-treated *T. b. brucei*⁷.

Although fluid-phase endocytosis appeared to be similar in *Tbr (R)* and *Tbr (S)*, it was possible that receptor-mediated endocytosis of macromolecules was reduced in TLf-resistant trypanosomes²⁴. To test this possibility, the rate of endocytosis of radiolabelled human transferrin was determined in *Tbr (R)* and *Tbr (S)* (Fig. 3a). We observed that both *Tbr (R)* and *Tbr (S)* are capable of receptor-mediated endocytosis of transferrin in a time-dependent and specific fashion (Fig. 3a). These results show that *Tbr (R)* cells are able to take up macromolecules by both receptor and fluid-phase mechanisms, suggesting that *Tbr (R)* cells either specifically fail to endocytose TLf or inhibit TLf activity intracellularly.

To distinguish between these two possibilities, uptake of ¹²⁵I-labelled TLf was studied in *Tbr (R)*, *Tbr (S)* and *T. b. brucei* cell lines. In contrast to the results with transferrin, *Tbr (R)* cells failed to accumulate radiolabelled TLf during the 30-min incubation (Fig. 3b). Incubation of *T. b. brucei* and *Tbr (S)* with ¹²⁵I TLf resulted in specific, time-dependent uptake (Fig. 3b). After 30 min, the amount of TLf taken-up by *Tbr (S)* cells was ~6-fold greater than by *Tbr (R)*

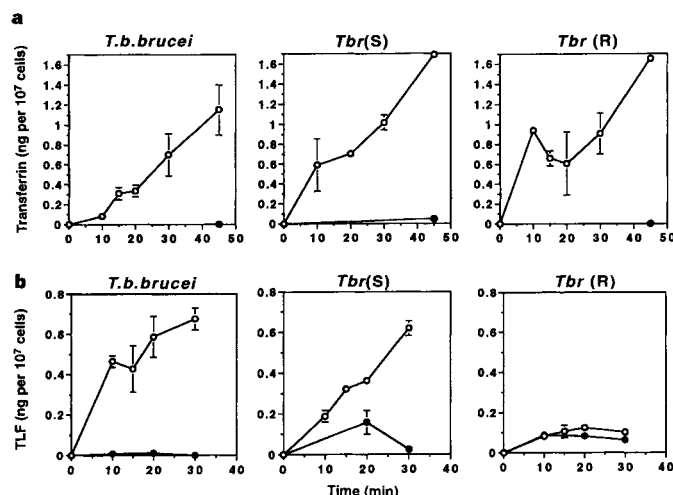


Figure 3 Receptor-mediated endocytosis of ^{125}I -labelled transferrin and TLF by *Tbr* (S) and *Tbr* (R) trypanosome lines. **a**, Purified *T. b. brucei*, *Tbr* (R) and *Tbr* (S) were incubated with ^{125}I -labelled transferrin at 37°C and uptake was monitored over time (white circles); results with unlabelled transferrin ($50\times$) are shown as black circles **b**, Purified *T. b. brucei*, *Tbr* (R) and *Tbr* (S) were incubated with ^{125}I -labelled TLF at 37°C and monitored over time (white circles) or with unlabelled TLF ($50\times$) (black circles). Each point was determined in triplicate (error bars, s.d.).

cells. The decreased accumulation of ^{125}I -labelled TLF by *Tbr* (R) cells was verified by *in situ* immunofluorescence using monoclonal antibodies raised against TLF apolipoproteins (Fig. 4). Unexpectedly, *Tbr* (R) cells bind TLF in the flagellar pocket region of the cells but fail to accumulate TLF intracellularly. Conversely, in *Tbr* (S) cells TLF is found not only in the flagellar pocket where binding occurs, but also in endocytic vesicles and is concentrated perinuclearly in the lysosome (Fig. 4).

We conclude that the VSG expressed on the trypanosome surface does not influence susceptibility to TLF-mediated lysis and that TLF-resistant *T. b. rhodesiense* express a high-affinity TLF-binding protein in the flagellar pocket. *T. b. rhodesiense* resistance to TLF-mediated lysis appears to be the result of a specific decrease in the endocytosis of receptor-bound TLF, which may be a general mechanism of *T. b. rhodesiense* resistance; however, this remains to be tested by using other *T. b. rhodesiense* isolates. The precise mechanisms involved in selectively restricting the endocytosis of receptor-bound TLF by *T. b. rhodesiense* are not understood. □

Methods

Trypanosome lysis assay. Lysis assays have been described⁸. A TLF-susceptible line of *T. b. brucei*, II^{Tat} 1.3, was used as a control in all experiments. Trypanosomes were grown to $1-5 \times 10^8$ cells ml^{-1} in Swiss CD-1 mice, infected blood was collected by cardiac puncture, and trypanosomes were isolated from it by DEAE chromatography^{25,26}. Per cent lysis was estimated by $40\times$ phase microscopy; one unit of TLF is taken as 50% lysis of *T. b. brucei*, (II^{Tat} 1.3) in 2 h at 37°C .

Fluid-phase endocytosis. Cells were incubated with 3 mg ml^{-1} tetramethylrhodamine-labelled dextran (Molecular Probes; $M_r = 10,000$; neutral charge) for 30 min at 37°C . Dextran is a bulk-phase endocytic marker used fluorescently to monitor uptake and endocytic vesicle integrity. Sample cells were subsequently treated with 4 units of TLF for 45 min at 37°C , control cells were mock-treated for the same time and temperature. Cells were fixed in 1% (v/v) formaldehyde (Toumisisin), washed three times with 0.37% (v/v) formaldehyde and resuspended in F12/FBS (F₁₂ (Gibco)/10% fetal bovine serum) (Fig. 1). The cell suspension was then smeared on slides, allowed to air-dry and mounted in 1:9 PBS/glycerol containing 0.1% phenylene diamine (Fisher Scientific) to prevent quenching. Slide specimens were viewed in a Leitz fluorescence

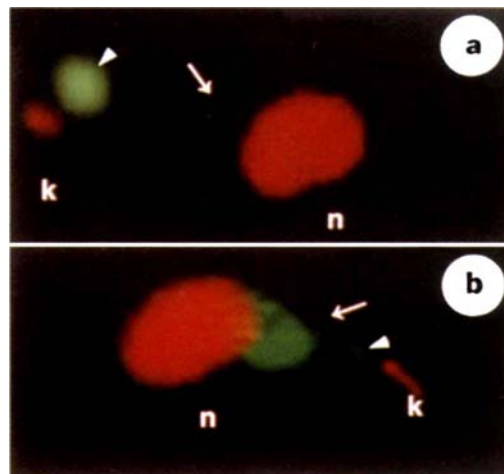


Figure 4 Immunolocalization of TLF in **a**, *Tbr* (R), and **b**, *Tbr* (S) cells. Trypanosomes were incubated with TLF for 15 min at 37°C and TLF was localized by immunofluorescence microscopy to the lysosomal region in *Tbr* (S) cells (arrow) but is restricted to the flagellar pocket (arrowhead) of *Tbr* (R) cells. No TLF is found in the lysosomal region of the *Tbr* (R) cells (arrow). Kinoplast (K) and nucleus (n) were stained with Hoescht dye.

microscope equipped with a Vario Orthomat II camera system and photographed using Kodak Ektachrome (EES 135-6) 800/1600 film (pushed to 1600 ASA in the development) (Eastman Kodak).

Receptor-mediated endocytosis. TLF and transferrin were radiolabelled using chloramine T as catalyst. Unincorporated ^{125}I was removed by filtration on a PD10 column (Pharmacia). Proteins were checked for aggregation and uniformity of label by SDS-PAGE followed by autoradiography. Uptake was determined as follows. Collected cells were incubated with $6.7 \mu\text{g ml}^{-1}$ ^{125}I -labelled transferrin or $3.3 \mu\text{g ml}^{-1}$ ^{125}I -labelled TLF in a modification of ref. 27. In brief, cells (1×10^8 cells per ml) were incubated in Baltz medium supplemented with 1% ovalbumin (chicken) and radiolabelled ligand at 37°C , plus or minus 50-fold unlabelled competitor. Aliquots were taken at each time point, washed five times in PBS plus 0.1% ovalbumin, and counted in a gamma counter.

In situ immunofluorescence. Cells were collected and then incubated with $5 \mu\text{g ml}^{-1}$ TLF at 37°C for 15 min and fixed in 8% EM-grade formaldehyde on ice for an hour. The cell suspension was centrifuged, washed and resuspended in F12/BSA. The cells were smeared onto microscope slides and air-dried, permeabilized with 0.1% Triton X-100 for 2 min, then washed and incubated with SF-11.4 monoclonal antibody raised against the 45K TLF subunit for 1 h at room temperature. Slides were washed three times in PBS and incubated with secondary goat anti-mouse fluorescein-conjugated antibody for 1 h at room temperature. Cells were washed and stained with 0.1% Hoescht dye in PBS and mounted in 1:9 PBS/glycerol with 0.1% phenylenediamine (Fisher). Slides were then viewed with a digital fluorescence microscope.

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Hrs-2 is an ATPase implicated in calcium-regulated secretion

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Associations between proteins present on neurotransmitter-containing vesicles and on the presynaptic membrane are thought to underlie docking and fusion of synaptic vesicles with the plasma membrane, which are obligate steps in regulated neurotransmission^{1–4}. SNAP-25 resides on the plasma membrane and interacts with syntaxin (a plasma membrane t-SNARE) and VAMP (a vesicle v-SNARE)^{1–9} to form a core protein complex thought to be an intermediate in a biochemical pathway that is essential for vesicular transport. We have now characterized a protein, Hrs-2, that interacts with SNAP-25. The binding of Hrs-2 to SNAP-25 is inhibited by calcium in the physiological concentration range that supports synaptic transmission. Furthermore, Hrs-2 binds and hydrolyses nucleoside triphosphates with kinetics that suggest that ATP is the physiological substrate for this enzyme. Hrs-2 is expressed throughout the brain and is present in nerve terminals. Moreover, recombinant Hrs-2 inhibits calcium-triggered ³H-noradrenaline release from permeabilized PC12 cells. Our results suggest a role for Hrs-2 in regulating secretory processes through calcium- and nucleotide-dependent modulation of vesicle-trafficking protein complexes.

Using the yeast two-hybrid approach¹⁰, we isolated a complementary DNA encoding a protein that interacts selectively with SNAP-25. The putative protein encoded by the rat clone is similar to Hrs, a growth-factor-induced phosphoprotein¹¹. The consistent sequence we obtained in the analysis of human and rat clones demonstrates that the previously published sequence of Hrs is either incorrect at the carboxy terminus or is a splice variant of the predicted protein presented here. The predicted open reading frame (ORF) from the rat cDNA contains domains with potential functional significance (Fig. 1a). A zinc-finger composed of eight cysteine residues is present in the amino-terminal half of the protein. A putative nucleotide-binding site is also present¹². The Hrs-2 protein sequence has two 28-amino-acid regions with the potential ($P = 0.74$ and $P = 0.97$, respectively) to form a coiled-coil

structure, as well as a glutamine- and proline-rich region. The domain structure suggests a role for Zn²⁺ ions, nucleotides, and coiled-coil mediated protein–protein interactions.

Immunoblotting using a polyclonal antibody raised against Hrs-2 bacterial fusion protein reveals a single band of relative molecular mass ~115K which is most abundant in brain (Fig. 1b). Brain postnuclear supernatant contains both Hrs-2 and SNAP-25, and incubation with anti-Hrs-2 antiserum, but not rabbit IgG, resulted in precipitation of both Hrs-2 and SNAP-25 (both antibodies recognize single bands on western analysis; Fig. 1b and c), providing evidence for this interaction *in vivo* (Fig. 1d). Direct and saturable binding of Hrs-2 to SNAP-25 was demonstrated using bacterially expressed fusion proteins (Fig. 2a). Recombinant Hrs-2 is able to bind Zn²⁺ ions based on binding of the recombinant protein to Zn²⁺-coupled agarose (data not shown). Although the binding of Hrs-2 to SNAP-25 remained saturable in the presence of Zn²⁺, the maximum binding was reduced about fourfold (Fig. 2a). Thus, the binding of Zn²⁺ to Hrs-2 may alter the conformation of Hrs-2 in such a manner that its affinity for SNAP-25 is decreased. Alternatively, Zn²⁺ may modulate the multimeric state of Hrs-2 (Fig. 3), or the effect of zinc may be unrelated to its binding to the zinc-finger of Hrs-2. The inhibition of Hrs-2–SNAP-25 binding by Zn²⁺, and the previously reported enhancement of neurotransmitter release induced by Zn²⁺ (refs 13, 14) suggest that the inhibition of Hrs-2–SNAP-25 binding may facilitate transmitter release.

In addition to Zn²⁺ ions, the zinc-finger or other domains of Hrs-2 may bind calcium, so we examined the effect of calcium on Hrs-2 binding to SNAP-25. Hrs-2 binding to SNAP-25 decreased with addition of increasing concentrations of free calcium (Fig. 2b). Barium was ~100 times less effective than calcium, and strontium had no effect on Hrs-2 binding to SNAP-25. The Hrs-2/SNAP-25 interaction is altered by calcium in the physiological concentration range that supports regulated secretion¹⁵.

As the Hrs-2 primary structure contains putative nucleotide-binding motifs, we investigated whether recombinant Hrs-2 could bind and hydrolyse nucleotides. Hrs-2 tagged with glutathione-S-transferase (GST) was purified by affinity chromatography and gel filtration. Two protein peaks containing full-length Hrs-2 immunoreactivity were obtained on the gel-filtration column: the position of one peak is consistent with monomeric protein whereas the other migrates at a position consistent with a multimer (Fig. 3a). Both peaks possess ATPase, GTPase and UTPase activity (Fig. 3b and c). The substrate-response curves for monomeric Hrs-2 ATPase, GTPase and UTPase activity were best fitted by single-site models (values for r^2 were 0.99, 0.99 or 0.98, respectively, where r^2 is the coefficient of variance) with apparent K_m values of 152 μ M, 123 μ M or 77 μ M, and K_{cat} values of 0.41, 0.18 or 0.46 min^{–1}, respectively (Fig. 3b). The nucleotidase activity of the oligomeric form of Hrs-2 demonstrates that ATP is the preferred substrate. The kinetic data for the oligomer were also best fitted by single-site models (r^2 values of 0.99, 0.99, 0.95, respectively) with apparent K_m values of 53, 487 or 61 μ M, and K_{cat} values of 2.64, 2.08 or 0.32 min^{–1}, respectively (Fig. 3c). These kinetic studies, and the fact that intracellular concentrations of ATP are higher than GTP and UTP, suggest that under physiological conditions ATP is the nucleoside triphosphate substrate for the Hrs-2 enzyme, and that the most active form of the enzyme is an oligomer. The K_m and K_{cat} values of Hrs-2 for ATP are similar to those reported previously for NSF, another oligomeric ATPase that interacts with other components of the secretory apparatus^{16–18}. ATP- γ S and GTP- γ S inhibited the ATPase activity of monomeric Hrs-2 with similar potency and in a monophasic manner, indicating the presence of a single nucleotide-binding site, which is consistent with the deduced amino-acid sequence (Fig. 3d, e). The ATPase activity of the oligomeric form was also inhibited by ATP- γ S, GTP- γ Ss, as well as EDTA (data not shown), suggesting that Hrs-2 requires Mg²⁺ for this enzyme activity.

To test whether Hrs-2 modulates exocytosis, recombinant Hrs-2

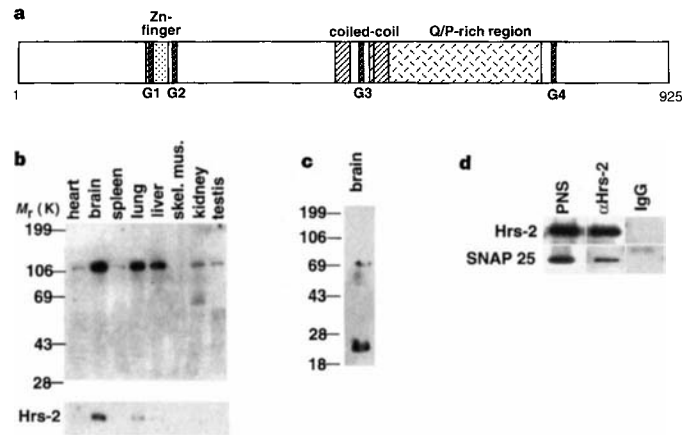


Figure 1 **a**, The domain structure of Hrs-2. The region surrounding the 8 cysteine residues that form two zinc-coordination domains (zinc-finger) is well conserved among a variety of proteins proposed to be involved in protein trafficking to the yeast vacuole (for example, Fab1, VPS 27, VPS 19, Vac1) or to the endosome in eukaryotic cells (for example, p162). The first three domains of the putative nucleotide-binding site are identical to the consensus GXXXXGK, RDET, DXXG (G1,G2,G3), whereas the fourth domain TQKXD (G4) is a variant found in some GTP-binding proteins¹². The G4 domain is thought to confer selectivity for the nucleotide, although some proteins specific for GTP completely lack the G4 domain^{12,25,26}. The spacing between the domains appears larger than usual and it is not clear whether the putative G4 domain we identified functions to coordinate

nucleotide binding^{25,26}. Two 28-amino-acid predicted coiled-coil domains (coiled-coil) and a glutamine- and proline-rich region (Q/P rich) are indicated. The accession number for this sequence is U87863. **b**, Top, immunoblot analysis of Hrs-2 in various rat tissues; bottom, lighter exposure of the identical blot shown above. Skel. mus., skeletal muscle. **c**, Immunoblot analysis of rat brain post-nuclear supernatant using the SNAP-25 antisera used in **d**. **d**, Immunoprecipitation of Hrs-2 and SNAP-25 from rat brain post-nuclear supernatant (PNS). Both Hrs-2 and SNAP-25 are present in rat brain PNS. Incubation with anti-Hrs-2 antiserum (α Hrs-2), but not rabbit IgG, resulted in precipitation of both Hrs-2 and SNAP-25.

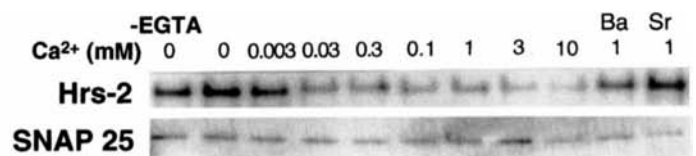
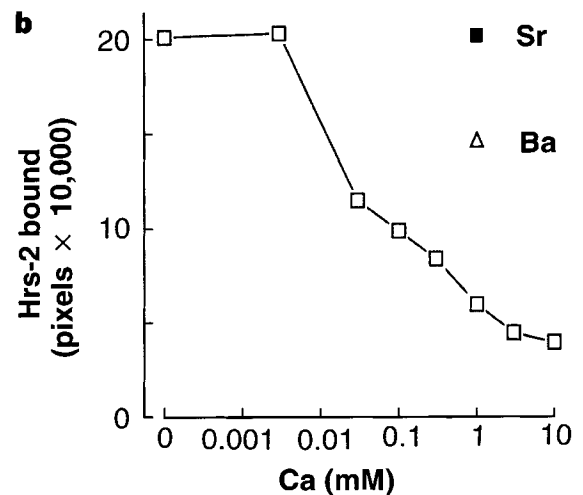
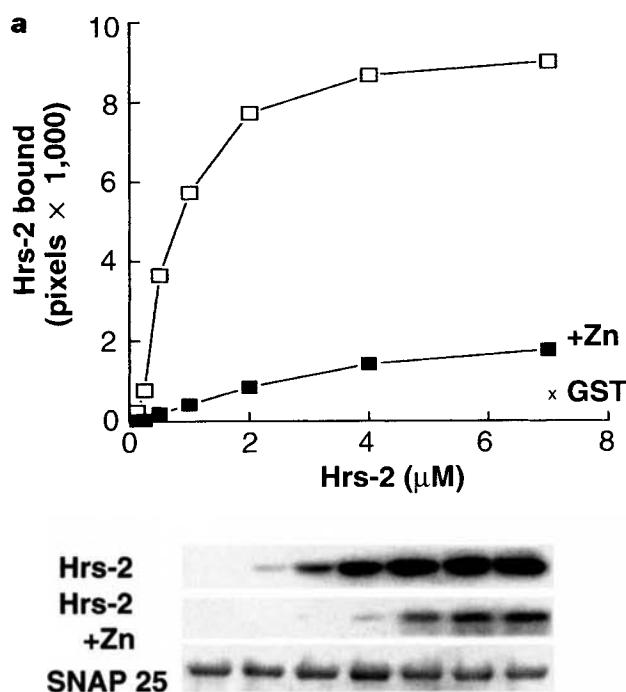


Figure 2 **a**, Hrs-2 binding to GST-SNAP-25 immobilized on glutathione-agarose beads and inhibition by Zn^{2+} . Pixel values ($\times 1,000$) for Hrs-2 were: 0.125 μ M, 238; 0.25 μ M, 767; 0.5 μ M, 1,652; 1 μ M, 5,724; 2 μ M, 7,728; 4 μ M, 8,677; 7 μ M, 9,012. Pixel values ($\times 1,000$) for Hrs-2 + Zn^{2+} were: 14, 20, 187, 413, 847, 1,422, 1,772. By quantifying the amount of Hrs-2 bound to SNAP-25 when one of the proteins was immobilized on glutathione-agarose beads and the other was soluble, we determined the stoichiometry of the interaction to be between 0.1–0.55:1 (Hrs-2:SNAP-25), depending on whether Hrs-2 or SNAP-25 was immobilized. **b**,

Binding of Hrs-2 to immobilized GST-SNAP-25 in the absence or presence of increasing concentrations of free calcium. The binding of Hrs-2 to GST-SNAP-25 did not differ in the absence or presence of EGTA (2 mM). As the concentration of free calcium was increased, the apparent binding of Hrs-2 to SNAP-25 decreased. Barium (1 mM) weakly inhibited, but strontium (1 mM) had no effect on the apparent binding of Hrs-2 to GST-SNAP-25. Pixel values ($\times 10,000$) for Hrs-2 were: 0 μ M (– EGTA), 20.13; 0 μ M, 20.32; 3 μ M, 20.31; 30 μ M, 11.95; 100 μ M, 9.713; 300 μ M, 7.974; 1 μ M, 5.80; 3 mM, 4.472; 10 mM, 3.574.

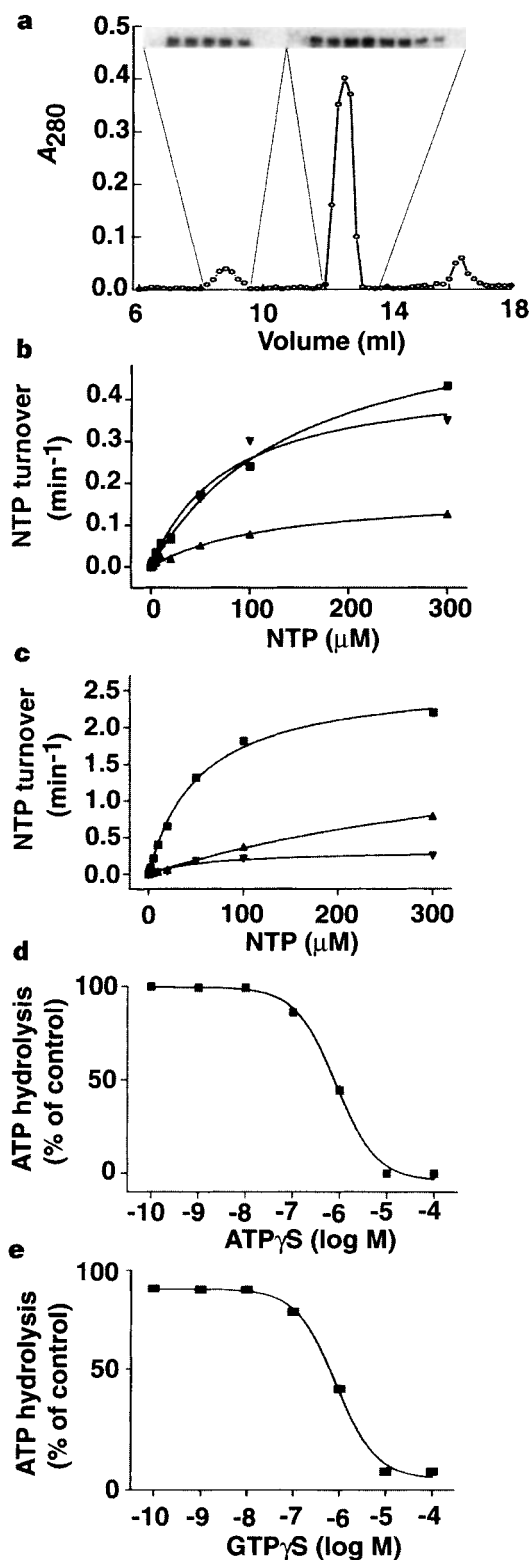


Figure 3 Hrs-2 has intrinsic nucleotidase activity. **a**, Recombinant Hrs-2 elutes from a gel filtration column in two peaks. Top, profile of fractions of eluate probed with anti-Hrs-2 antibody; bottom, protein elution profile (A_{280} , absorbance at 280 nm) from the gel-filtration column. **b**, NTPase activity of monomeric Hrs-2 (right column peak in **a**). ■, ATPase; ▲, GTPase; ▼, UTPase. **c**, NTPase activity of oligomeric Hrs-2 (left column peak in **a**). ■, ATPase; ▲, GTPase; ▼, UTPase. **d** and **e**, Inhibition of the ATPase activity of monomeric Hrs-2 by ATP- γS and GTP- γS . ATPase activity was assessed in the presence of 1 μM ATP with various concentrations of ATP- γS (■) and GTP- γS (▲). The IC_{50} values for ATP- γS and GTP- γS are 815 nM and 843 nM, respectively.

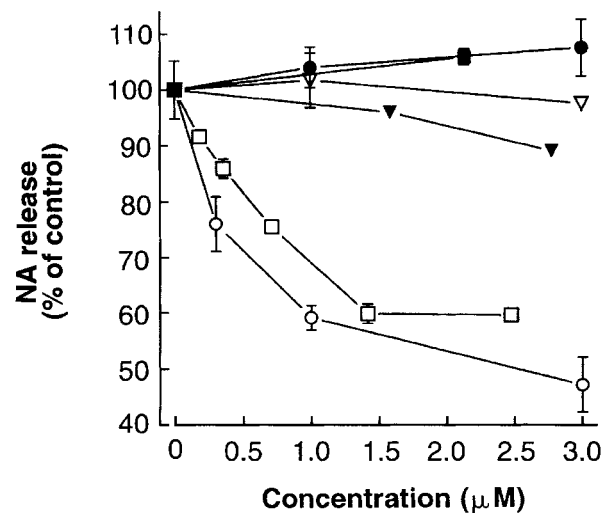


Figure 4 Hrs-2 (□) dose-dependently and saturably inhibits the release of ^3H -noradrenaline (NA) from permeabilized PC12 cells. Secretion is presented as the percentage of ^3H -NE released of the total ^3H added in each individual triggering reaction (^3H in supernatant/ ^3H in supernatant + ^3H in pellet) compared to the maximum stimulated secretion produced when adding all reaction components in the absence of recombinant proteins. Control proteins, GST (■), GST- α -catenin (▼), GST-SNAP-25 (●), and GST- α SNAP (▽) had minimal effect on secretion at molar concentrations equal to or greater than those used for Hrs-2. GST-syntaxin (○) inhibited secretion with similar efficacy to Hrs-2.

was added to a permeabilized PC12 cell reconstitution system for MgATP-dependent, calcium-regulated, ^3H -noradrenaline (NA) secretion (Fig. 4)¹⁹. Hrs-2 markedly inhibited secretion in a dose-dependent and saturable manner. Four control proteins, GST, GST-SNAP-25, GST- α SNAP^{1,2,6,8} and GST- α -catenin (a 128K protein that is not involved in neurotransmission but is implicated in cell-cell adhesion)²⁰, were without effect on ^3H -NA secretion at molar concentrations equal to or greater than those at which Hrs-2 inhibited secretion (Fig. 4). Recombinant syntaxin 1a (cytoplasmic domain) inhibited secretion to an extent similar to Hrs-2 (Fig. 4). The inhibition observed when Hrs-2 is added in the priming step may reflect an action of Hrs-2 before ATP hydrolysis, but we cannot rule out the possibility that Hrs-2 binds to its target during the priming step and has its effect on subsequent reactions²¹. These data provide a functional demonstration of the ability of Hrs to regulate the secretory apparatus.

We have identified a new ATP-preferring nucleotidase that associates with SNAP-25, a component of the protein complexes thought to underlie vesicle docking and fusion. Binding of the recombinant protein to SNAP-25 is reduced by addition of calcium in the concentration range that supports neurotransmission¹⁵. Hrs-2 dose-dependently inhibits calcium-triggered ^3H -NA release from permeabilized PC12 cells. We do not yet know whether Hrs-2 prevents SNAP-25 from interacting with other proteins involved in secretion, or perhaps Hrs-2, by virtue of its ATPase activity, is involved in the formation or dissolution of protein complexes^{2,3,6,8}. We suggest that Hrs-2 plays a regulatory role both in docking and/or activation through its ATPase and in later stages of secretion, possibly membrane fusion, as a result of calcium-regulated interactions with SNAP-25 (refs 19, 21).

Methods

Two-hybrid screen and cloning. The entire coding region of mouse SNAP-25b (ref. 9) was amplified in the polymerase chain reaction (PCR) using primers (forward: 5'-CCG AAT TCA TGG CCG AGG ACG CAG ACA-3';

reverse: 5'-CCG TCG ACT AAC CAC TTC CCA GCA TCT-3') containing internal *EcoRI* and *SaII* restriction sites and inserted in-frame with the Gal4 binding domain in the pGBT9 vector (from S. Fields) to create pGBT9/SNAP-25. All constructions were verified by sequencing (Sequenase, USB). A human brain cDNA library inserted downstream of the GAL4 activation domain in the pGAD10 vector was purchased from Clontech. Yeast (HF7c strain; Clontech) were cotransformed²² with the pGAD/library and pGBT9/SNAP-25 (4×10^6 colonies), plated on agar containing yeast nitrogen base (6.7 g l⁻¹; Difco), adenine (0.054 mM), lysine (0.165 mM), dextrose (2%) and 3-amino-1,2,4-triazole (10 mM; Sigma) and stored at 30 °C in the dark. On the 16 positive clones recovered, multiple cotransformations were done with the resultant candidate DNA and the pGBT9 plasmid alone, pGBT9/SNAP-25 and pGBT9/lamin. Clones that reacted positively for β -galactosidase with pGBT9/SNAP-25, but not by themselves or with the control plasmids, were considered for further study. A rat brain library (AZAP II; Stratagene) was screened with a random-primed ³²P-labelled insert from the human Hrs-2 clone, enabling a single clone to be isolated which contained the full coding region, including a consensus translational start site²³. An additional clone included an in-frame stop codon upstream of the first methionine, thus confirming its position. The coding sequence of the rat clone encodes a 925-amino-acid peptide with a predicted relative molecular mass (M_r) of 106,764K. The human clone initially recovered in the two-hybrid screen contained all of the rat clone apart from the N-terminal 149 amino acids.

Protein purification NTPase assay, binding and immunoprecipitation. Recombinant GST-Hrs-2 was purified by affinity chromatography, cleaved from the GST tag using thrombin, and size-fractionated by gel filtration (Superose-12) using a buffer consisting of 50 mM Tris, 500 mM NaCl and 0.05% Tween 20, pH 7.8. NTPase reactions (100 μ l) contained Hrs-2 (10 μ l of Superose-12 column fractions pooled from monomer and oligomer peaks), unlabelled NTPs at various concentrations, 1.5–2.0 μ Ci of the corresponding [γ -³²P]NTPs, 1 mM MgCl₂, 0.1 mM EDTA, 0.2% (w/v) BSA and 50 mM Tris-HCl, pH 7.4. Reactions were run for 20 min at 25 °C and then terminated by addition of an ice-cold slurry containing 5% (w/v) activated charcoal and 50 mM NaH₂PO₄, pH 2.0. Radioactivity in supernatants of reaction mixtures was determined by scintillation counting. Blank values were determined using column-elution buffer in the reaction mixture. Steady-state NTPase activity of Hrs-2 is expressed as molar turnover number (mol NTP hydrolysed per mol Hrs-2 per min). Data shown are the mean $\pm$ s.d. of one representative experiment done in triplicate. Very similar results were obtained in 2–3 independent experiments with different preparations of Hrs-2.

For binding experiments, either increasing concentrations (0.11–7 μ M) of Hrs-2 were incubated with immobilized GST-SNAP-25 (0.3 μ M, 4 °C for 60 min) in the presence or absence of Zn²⁺ (1 mM) or with immobilized GST, or a single concentration of Hrs-2 (2 μ M) was incubated with immobilized GST-SNAP-25 in the presence of various free calcium concentrations. After washing, beads were boiled in SDS-containing sample buffer and proteins were separated by SDS-PAGE. After transfer to nitrocellulose, blots were probed with anti-Hrs-2 antibodies and ¹²⁵I-labelled secondary antisera. Hrs-2 was visualized and quantified using a phosphorimager.

Immunoprecipitation was achieved by incubating detergent-solubilized (Triton X-100, 1%) rat brain post-nuclear supernatant with affinity-purified anti-Hrs-2 antiserum or rabbit IgG that had been covalently bound to protein A-Sepharose beads with dimethylpimelidate for 16 h at 4 °C. Samples were washed three times and resuspended in 15 μ l sample buffer, separated by SDS-PAGE (10%), transferred to nitrocellulose, and probed with antibodies. Proteins were visualized using chemiluminescence (Amersham ECL).

Permeabilized cell secretion assay. PC12 cells were maintained as described²⁴ and secretion assayed¹⁹. Exogenous protein was added into the priming (and removed before triggering) or triggering reactions to examine its effect on each secretion step. We were unable to assess whether Hrs-2 had an effect in the triggering reaction as this reaction step is highly sensitive to the effects of protein mass and we observed nonspecific effects of our high- M_r control protein (α -catenin) added at equivalent concentrations. GST protein had no effect on secretion at molar concentrations 4 times the highest concentration of Hrs-2 tested. Six secretion experiments were done using either 6XHIS-Hrs-2 (3) or GST-Hrs-2 (3). Dose-response curves to ATP in the presence and absence of Hrs-2 revealed an inability of increased ATP to

overcome the inhibition of secretion by Hrs-2, suggesting that the inhibition of secretion is not simply due to degradation of necessary ATP by Hrs-2. Each condition was tested in duplicate.

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pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*

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Members of the Wnt/Wingless (Wg) family of signalling proteins organize many aspects of animal development by regulating the expression of particular target genes in responding cells^{1–6}. Recent biochemical studies^{7–9} indicate that the vertebrate HMG-domain proteins Lef-1 and XTcf-3 can physically interact with β -catenin, a homologue of *Drosophila* Armadillo (Arm), the most downstream component known in the Wnt signal transduction pathway^{10,11}. However, these studies do not address whether the endogenous Lef/Tcf family members are required *in vivo* to transduce Wnt signals. Using genetic methods in *Drosophila*, we define a new segment polarity gene, *pangolin* (*pan*), and show that its product is required *in vivo* for Wg signal transduction in embryos and in developing adult tissues. In addition, we show that *pan* encodes a Lef/Tcf homologue and provide evidence that its protein product binds to the β -catenin homologue Armadillo *in vivo*. Finally, we demonstrate that Pan functions downstream of

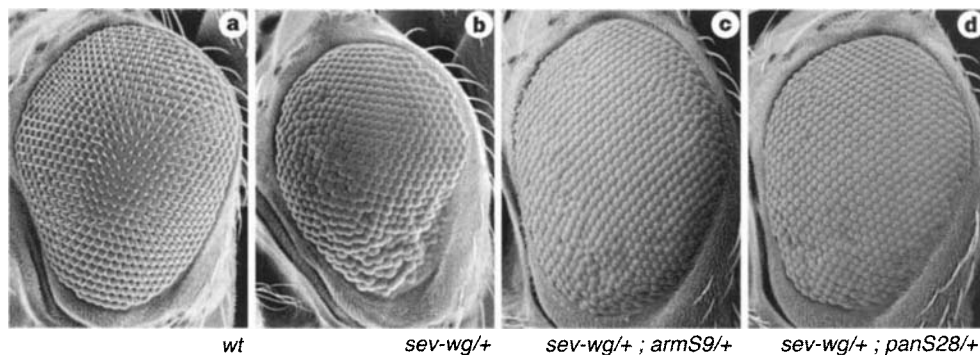


Figure 1 Eye phenotypes of wild-type (a), *sev-wg* (b), *sev-wg, arm^{S9}/+* (c) and *sev-wg, pan^{S28}/+* (d) animals. Scanning electron micrographs are shown, anterior is to the left. The smooth, regular arrangement of the ommatidial facets is disrupted by the expression of a *sev-wg* transgene in a dosage-dependent

manner. The number of photoreceptor cells is reduced and interommatidial bristles are lacking (see also ref. 32). Strong and dominant suppression of the rough eye phenotype is observed in *arm^{S9}/+* and *pan^{S28}/+* heterozygous animals.

Arm to transduce the Wg signal. Thus, our results indicate that Pan is an essential component of the Wg transduction pathway and suggest that it acts directly to regulate gene transcription in response to Wg signalling.

To identify new components of the Wg signal transduction pathway we performed a screen for dominant suppressors of the rough eye phenotype caused by a transgene that drives ectopic expression of *wg* during eye development (Fig. 1a, b). Four strong suppressors were found (Fig. 1c, d) that fall into two complementation groups. Genetic mapping revealed that *Sup^{S8}* and *Sup^{S9}* are recessive alleles of *arm* (ref. 10), and *Sup^{S25}* and *Sup^{S28}* are recessive alleles of a locus on the fourth chromosome that we designate *pangolin* (*pan*). We subsequently isolated a stronger allele of *pan*, *pan^{ER1}*, which shows strong genetic interactions when trans-heterozygous with either the *arm^{S8}* or *arm^{S9}* mutation, causing adult phenotypes characteristic of reduced *wg* activity (see Methods and below). These results suggest that *pan*, like *arm*, encodes a component of the Wg transduction pathway, and raise the possibility that these components may physically interact.

Recently, the nuclear HMG-domain proteins Lef-1 and XTcf-3 were shown to bind directly to β -catenin, the vertebrate homologue of Arm (refs 7–9). To examine the possibility that *pan* encodes a Lef-1/XTcf-3-like protein we searched for a *Drosophila* Lef/Tcf homologue. Using degenerate primers that discriminate between the HMG-domain sequences of Lef-1/Tcf-1 and those of Sox- and other HMG-domain-family members, a sequence was identified that encodes an HMG domain very similar to that of Lef-1 and XTcf-3 (Fig. 2a). *In situ* hybridization revealed that the corresponding gene is located at the base of chromosome 4. This site is deleted in *Df(4)M^{62f}*, which does not complement mutations in *pan*. Molecular analysis of genomic cosmid clones showed that this gene is located just upstream of and distal to *cubitus interruptus* (*ci*) and is transcribed in the opposite orientation¹². It is widely expressed in the embryo, in a pattern that is segmentally modulated (Fig. 2b). To demonstrate that this gene corresponds to *pan*, we determined its entire open reading frame and sequenced the coding region of the *pan^{S25}* and *pan^{S28}* alleles (Fig. 2a). For each allele a single nucleotide change was detected that mutates a glutamic acid residue of the N-terminal region of the putative protein product into a positively charged lysine residue. These results indicate that *pan* encodes a Lef-1 homologue that, like Arm, is involved in Wg signal transduction.

The binding of Lef-1 to β -catenin is mediated by its N-terminal 56 amino acids⁷. Because *pan^{S25}* and *pan^{S28}* encode amino-acid substitutions in the corresponding domain of Pan, we tested whether the wild-type and mutant N-terminal portions of Pan (amino acids 1–130) can bind *in vitro* to purified vertebrate β -catenin. All three Pan proteins can bind to β -catenin (Fig. 2c), but

the efficiency of binding appears to be reduced fivefold for *Pan^{S25}*[1–130] and *Pan^{S28}*[1–130]. These results indicate (1) that Pan can physically interact with the Arm homologue β -catenin and (2) that mutations in this domain that compromise Wg signalling *in vivo* have a corresponding deleterious effect on the affinity of the binding interaction assayed *in vitro*. We interpret these findings, which reinforce our observations of genetic interactions between *arm* and *pan* alleles, as evidence that Wnt/Wg signal transduction normally depends on functional interactions between members of the Lef/Tcf class of transcription factors and β -catenin/Arm proteins.

Wg plays multiple roles during development, organizing cell proliferation and cell fates in different tissues¹¹. To test whether Pan is a general mediator of Wg signalling we used the *pan^{ER1}* mutation, which is temperature sensitive, to assay the requirement for *pan* activity in various developmental contexts. *pan^{ER1}* homozygous animals are viable at 18 °C but die during larval or pupal stages if raised at 29 °C. At intermediate temperatures, homozygous animals display defects characteristic of reduced *wg* activity (Fig. 3). During larval development, Wg organizes antennal and ventral leg structures^{13,14}. Reduction of *wg* activity causes truncated antennae and double dorsal legs^{14–16}. The same phenotypes are observed in *pan^{ER1}* animals (Fig. 3a, b). Wg signalling plays at least two distinct roles during wing development. Early reduction of *wg* activity results in a wing-to-notum transformation, indicating a requirement for Wg in defining the wing blade primordium^{17,18}, whereas later reductions cause the loss of wing margin and adjacent tissue, indicating its subsequent role in specifying the wing margin and organizing wing blade development^{6,14,19–21}. As shown in Fig. 3, *pan^{ER1}* adults show both classes of phenotypes at high frequency. Finally, Wg signalling distinguishes between sternite and ventral pleura development in the adult abdomen²². Like *wg^{ts}* mutants, *pan^{ER1}* animals show extra pleura tissue at the expense of sternite structures (Fig. 3d). In sum, *pan* is required for all of the Wg signalling events that we have assayed in developing adult tissues. We also observe the same classes of phenotypes in trans-heterozygous *pan^{S28}/+*, *wg^{CE7}/+* and *pan^{ER1}/+*, *arm^{S9}/+* animals (not shown), supporting our argument that Wg signal transduction depends on direct interactions between Pan and Arm.

To assay further the requirement for *pan* activity in Wg signal transduction, we identified a loss-of-function allele (*l(4)13*, now termed *pan¹³*) among a collection of X-ray-induced 4th chromosomal lethals²³ by its failure to complement *pan^{ER1}*. *pan¹³* does complement all alleles of the neighbouring *ci* locus, with the exception of *ci^D* (ref. 12). Thus *ci^D* is mutant for two neighbouring loci, *ci* and *pan*. Indeed, *in situ* hybridization experiments with *pan* probes revealed that in *ci^D/+* embryos, *pan* transcripts are detected in a pattern indistinguishable from that of *ci* transcripts, providing

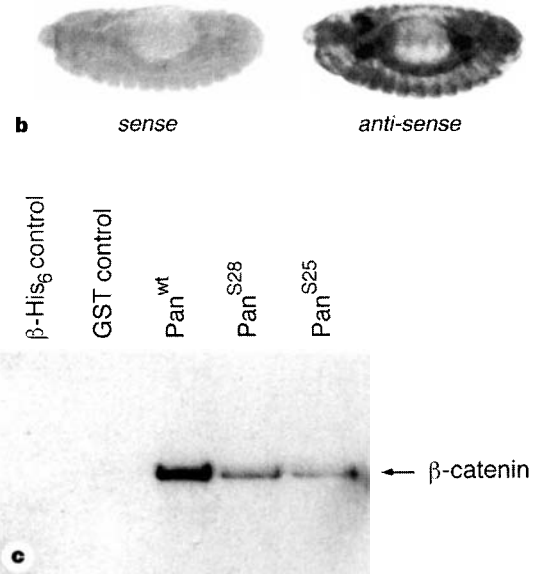
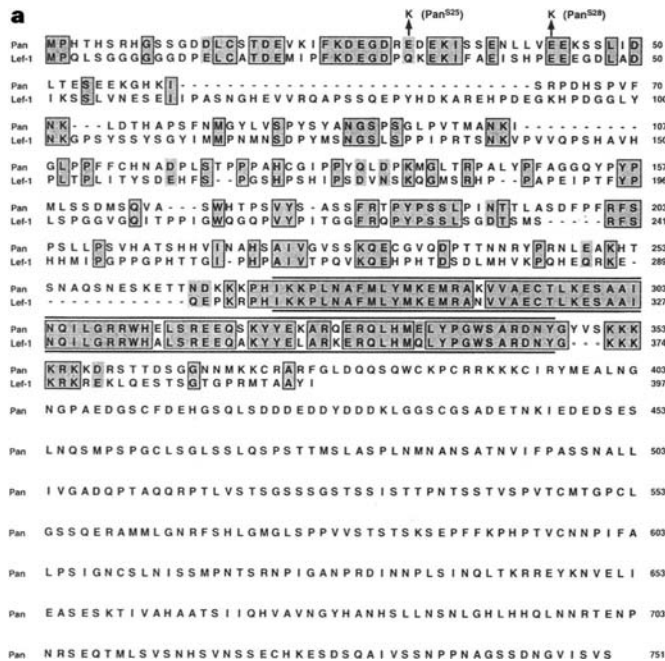


Figure 2 a, Amino-acid sequence of the putative Pan protein. The sequence of mouse Lef-1 is shown underneath³⁴. Identical residues are shaded and boxed, conserved residues are shaded. The HMG-domain (between lines) shows 88% identical residues. The N-terminal region of Pan is also conserved between Pan, Lef-1 and XTcf-3. The sequence just C-terminal to the HMG-domain (370 to 395) bears a highly conserved cluster of residues compared with the *Caenorhabditis elegans* Pop-1 gene product³⁵ (not shown). The amino-acid replacements found in the *pan*^{S25} and *pan*^{S28} mutants are indicated on top. **b**, Expression of *pan* during embryogenesis. *In situ* hybridization with digoxigenin-labelled sense (control) and anti-sense (experimental) RNA probes derived from our *pan* cDNA. The anti-sense probe reveals relatively ubiquitous distribution of *pan* transcripts throughout embryogenesis. The embryo shown has completed germ band retraction and

shows *pan* transcripts in a segmentally modulated pattern, with high accumulation in the visceral mesoderm and endoderm of the gut, as well as in part of the procephalon. **c**, Interaction of wild-type and mutant Pan with β-catenin. The N-terminal domains (1–130) of wild-type Pan, Pan^{S25} and Pan^{S28} were produced as GST fusion proteins in *E. coli* and equal amounts (2 μg) were incubated with recombinant His-tagged β-catenin (4 μg) in a volume of 200 μl. Complexes were purified on glutathione-agarose beads and subjected to immunoblot analysis with anti-β-catenin antibodies. To control for unspecific binding, β-catenin-His₆ protein was incubated without GST-Pan protein (β-His₆ control) or a GST epitope without Pan sequences was used (lane GST control). As quantified by densitometry, the mutant Pan proteins bind four-to-fivefold less β-catenin compared to wild-type.

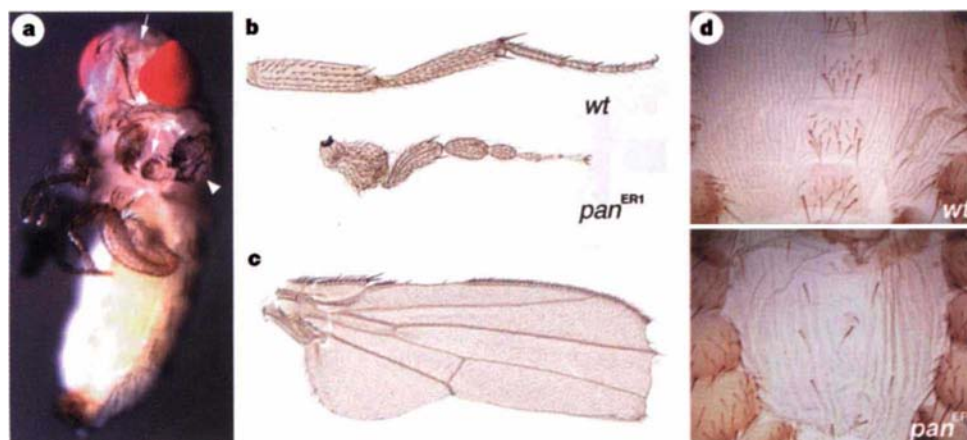


Figure 3 Adult *pan* phenotypes. **a**, Pharate adult of the genotype *pan*^{ER1}/*pan*¹³. Antennae and legs are crippled or absent (arrows), the wing is transformed into a duplicated notum (arrow head). **b**, Typical leg defect of *pan*^{ER1} homozygous animals: a strong dorsalization results in the loss of dorso-ventral polarity of the

leg pattern. **c**, Wing notches of a *pan*^{ER1} homozygote. Notches are found in 20 to 30% of the animals raised at 25°C. **d**, Ventral aspect of a wild-type and a *pan*^{ER1} mutant abdomen. Sternite structures are affected in all animals. Typically, sternite bristles are lost and sternite tissue is replaced by pleura.

additional evidence that the molecular lesion of *ci^D* disrupts both genes (data not shown). It is a curious coincidence that *pan* and *ci* are adjacent genes, as *ci* encodes a transcription factor which is essential for transducing all known examples of Hedgehog (Hh) signalling^{24–28}, whereas our present evidence suggests an equivalent role for Pan in Wg signal transduction. The fact that the *ci^D* mutation abolishes both activities also calls for a reassessment of genetic epistasis experiments in which this allele is used to assay the relationship between Hh and Wg signalling.

Embryos homozygous for *pan¹³* display a segment polarity phenotype (Fig. 4a, b) in which the larval epidermis forms a lawn of ventral denticles and lacks naked cuticle between the segmental denticle belts, similar to *arm* mutants¹⁰, leading us to designate this locus *pangolin*. This phenotype is equally strong in *pan¹³/Df(4)M^{62f}* embryos, thus *pan¹³* behaves like a null allele. The zygotic *pan* mutant phenotype is not as extreme as that of *wg^{ts}* embryos because the individual denticles retain their correct polarity. However, it appears identical to the phenotype of *wg^{ts}* embryos that are shifted to the restrictive temperature at 7 h of development (Fig. 4c; ref. 29). Hence, the less extreme phenotype associated with *pan¹³* embryos may reflect partial rescue of the true *pan* null phenotype by maternal

pan product that perdures and allows Wg to be transduced during early embryonic stages.

To test whether the Wg signal transduced by Arm can bypass the Lef/Tcf homologue encoded by *pan*, we used the suppression of ventral denticles as an assay for Wg signalling^{6,30}. A constitutively active form of Arm, Δ Arm (ref. 6), was expressed indiscriminately during embryogenesis in different mutant backgrounds. In wild-type control embryos Δ Arm expression results in a 'naked' phenotype in which the denticles normally positioned in the anterior portion of each segment are replaced partially or completely by naked cuticle (Fig. 4d; ref. 6). In *pan¹³* mutant embryos, however, no naked cuticle can be observed, despite high levels of Δ Arm expression (Fig. 4e). As a control, we also applied this test to embryos with a segment polarity phenotype caused by the loss of *ci* activity which acts upstream of *wg* (ref. 28). As in wild-type, the formation of ventral denticles in *ci^{Cell}* mutant embryos is suppressed by Δ Arm activity (Fig. 4f). Thus, in the absence of Pan, Arm activity is blocked and cannot cause a biological Wg response *in vivo*. We infer from these results that there is an absolute requirement for Pan in the transduction of the Wg signal.

In summary, we describe a new *Drosophila* segment polarity gene, *pangolin*, and show that it encodes a homologue of the vertebrate transcription factor Lef-1/XTcf-3. In addition, we establish that *pan* activity is essential for mediating Wg signal transduction *in vivo* and present genetic and molecular evidence that its transducing activity depends on direct interactions with the β -catenin homologue Arm. Finally, we show the results of a genetic epistasis experiment which indicate that Pan acts downstream of Arm, previously the most downstream component of the Wg transduction pathway identified. Thus, we propose that Pan is an essential component of the Wg transduction pathway which binds to, and regulates the transcription of, target genes in response to the Wg-dependent activation of Arm. □

Methods

Isolation of *pan* alleles. A *sev-wg* transgene was constructed based on the P-element plasmid *sev^{S11}* (ref. 31) containing two copies of the sevenless enhancer. The resulting phenotype is stronger than that described by Cadigan *et al.* (ref. 32) who used a different construct: *sev-wg* transformants not only lack interommatidial bristles, they also display a rough eye phenotype which is dosage sensitive. Ethylmethane sulphonate (EMS)-treated males were crossed to *sev-wg* females. Among 2×10^5 progeny screened for enhancers and suppressors of the rough eye phenotype *pan^{S25}* and *pan^{S28}* were identified. Both alleles are homozygous viable. Suspecting that these alleles might act in a slightly dominant-negative manner we performed a second screen for revertants of *pan^{S28}* that fail to suppress the *sev-wg* phenotype consistently. This led to the isolation of *pan^{ER1}*, which was found to be temperature-sensitive. Most stock centres only carry the weak, EMS-induced *pan* allele, *l(4)13a*, isolated by Hochmann³³. The original *l(4)13 (pan¹³)* allele, described by Hochmann 7 years earlier²³, was retrieved from the backup collection of the Bowling Green Stock Center.

Cloning of *pan*. Primary embryonic cDNA served as substrate for fully degenerate polymerase chain reaction (PCR) primers corresponding to amino acids 304–311 and 364–371 of mouse Lef-1 (ref. 34). The resulting fragment was used to isolate genomic and cDNA clones from various embryonic cDNA libraries. The full-length sequence of the *pan* ORF was assembled from several partial cDNA clones. The entire ORF of the mutant alleles *pan^{S25}* or *pan^{S28}* was determined by isolating mRNA from homozygous embryos followed by reverse-transcribed PCR (RT-PCR). *pan* has a complex intron/exon structure that extends over more than 20 kb. The 5' end of our longest cDNA maps to position +10 on the genomic map of *ci* (ref. 12).

Binding studies. The first 400 nucleotides of the wild-type and mutant *pan* ORFs were amplified by PCR, cloned into pGEX2T and sequenced to verify that they are intact. Recombinant fusion proteins were isolated from *Escherichia coli* lysates by using glutathione-agarose columns, dialysed to 10 mM Tris-HCl pH 8.0, and quantified by gel electrophoresis and photospectrometry. Binding assays with β -catenin-His₆ were carried out as described⁷.

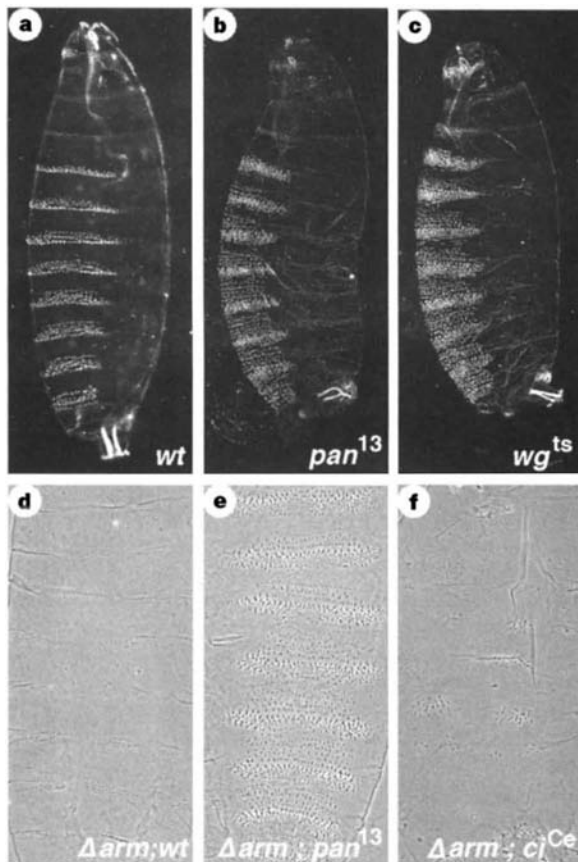


Figure 4 Embryonic *pan* phenotype and epistasis analysis. **a–c**, Cuticle preparations of larvae derived from wild-type (**a**), *pan¹³* (**b**) and *wg^{ts}* (**c**) embryos. The ventral epidermis of wild-type larvae displays regular denticle belts, spaced by naked cuticle. No naked cuticle is observed in *pan¹³* animals. The *wg^{ts}* embryo shown in (**c**) was shifted to restrictive temperature at 7 h of development. **d–f**, The Wg signal transduction pathway was activated by ubiquitous expression of a constitutively active form of Arm (Δ Arm) under the control of a *hs-Gal4* driver. In wild-type (**d**) and *ci^{Cell}* mutant (**f**) embryos, ventral denticles are replaced by naked cuticle. In *pan¹³* embryos, the lawn phenotype cannot be reversed by the constitutive activity of Δ Arm (**e**). Larvae were selected for (**d**) and (**f**) that only show a partial 'naked' phenotype, such that remaining lateral denticles serve as landmarks.

Epistasis experiments. Δ Arm was expressed using a *hs-Gal4* driver line in combination with a *UAS- Δ arm* transgene⁶. Embryos were heat-shocked two or three times at 37 °C for 30 min with a recovery time of 2 to 3 h at 25 °C between heat shocks. To perform this experiment in *pan¹³* or *ci^{Cell}* mutant backgrounds *yw, hs-Gal4/+*, *pan¹³* (or *ci^{Cell}*)/*Dp(1;4)1021[y⁺]* females were crossed to *yw, UAS- Δ arm/+*, *pan¹³* (or *ci^{Cell}*)/*Dp(1;4)1021[y⁺]* males, and the progeny were subjected to the same heat-shock regime. *y* mutant cuticles were identified by scoring denticles, beard or residual lateral denticles. In the case of *pan¹³*, we never observed a *y* mutant larva that showed reversal of the lawn phenotype. For the embryo shown in Fig. 4e, the experiment was repeated with progeny of *UAS- Δ arm/UAS- Δ arm, pan¹³/+* females crossed to *hs-Gal4/hs-Gal4, pan¹³/+* males.

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Crystal structure of the anthrax toxin protective antigen

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Protective antigen (PA) is the central component of the three-part protein toxin secreted by *Bacillus anthracis*, the organism responsible for anthrax¹. After proteolytic activation on the host cell surface, PA forms a membrane-inserting heptamer that translocates the toxic enzymes, oedema factor and lethal factor, into the cytosol^{2–4}. PA, which has a relative molecular mass of 83,000 (M,

83K), can also translocate heterologous proteins, and is being evaluated for use as a general protein delivery system^{5,6}. Here we report the crystal structure of monomeric PA at 2.1 Å resolution and the water-soluble heptamer at 4.5 Å resolution. The monomer is organized mainly into antiparallel β -sheets and has four domains: an amino-terminal domain (domain 1) containing two calcium ions and the cleavage site for activating proteases; a heptamerization domain (domain 2) containing a large flexible loop implicated in membrane insertion; a small domain of unknown function (domain 3); and a carboxy-terminal receptor-binding domain (domain 4). Removal of a 20K amino-terminal fragment from domain 1 allows the assembly of the heptamer, a ring-shaped structure with a negatively charged lumen, and exposes a large hydrophobic surface for binding the toxic enzymes. We propose a model of pH-dependent membrane insertion involving the formation of a porin-like, membrane-spanning β -barrel.

Protective antigen, named for its use in vaccines, is a long, flat molecule of dimensions $100 \times \sim 50 \times 30$ Å (Fig. 1b). Domain 1 (residues 1–258) comprises a β -sandwich with jelly-roll topology, several small helices, and a pair of adjacent calcium ions coordinated by residues in a variant of the EF-hand motif (Fig. 2a, b). Domain 2 (residues 259–487) has a β -barrel core with modified Greek-key topology and elaborate excursions, including a large flexible loop between strands $2\beta_2$ and $2\beta_3$ that is implicated in membrane insertion (Fig. 2c). Domain 3 (residues 488–595) has a four-stranded mixed β -sheet, two smaller sheets and four helices (Fig. 2d); it adopts the same fold as ferredoxins and resembles domain A of toxic-shock-syndrome toxin-1 (ref. 7). Domain 4 (residues 596–735) has an initial hairpin and helix, followed by a β -sandwich with an immunoglobulin-like fold (Fig. 2e). Domains 1, 2 and 3 are intimately associated, but domain 4 has limited contact with them.

Homologues of PA have been found in several spore-forming Gram-positive bacteria (for example, the iota-b toxin in *Clostridium perfringens*⁸ and the vegetative insecticidal protein (VIP1) toxins in *Bacillus cereus* and *B. thuringiensis*⁹) and share the ability to translocate toxic enzymes into the host cytosol. High sequence similarity over the first 600 residues and strictly conserved calcium-binding motifs indicate that the homologues share the same folds for domains 1, 2 and 3 (Fig. 1c).

The steps in host-cell intoxication are illustrated in Fig. 1a. Studies with blocking antibodies¹⁰, proteolytic fragments¹¹ and deletion mutants¹² implicate domain 4 in host-cell binding. Within the immunoglobulin-like fold, a 19-residue, highly accessible loop (between strands $4\beta_9$ and $4\beta_{10}$) is analogous to the antigen-binding CDR3 loop of antibodies and the receptor-binding loop of diphtheria toxin¹³. The loop is a good candidate for mediating the interaction between PA and the receptor, as short carboxy-terminal deletions that compromise the integrity of this loop eliminate cell binding (Fig. 2e).

Proteolytic activation by the cell-surface protease furin occurs in a surface loop within domain 1, releasing an N-terminal 20K fragment, PA₂₀ (residues 1–167), which plays no further role in intoxication. PA₂₀ forms an integral part of domain 1 (Fig. 2a), and dissociation requires the rupture of a β -sheet (between strands $1\beta_2$ and $1\beta_{13}$) and exposure of a large hydrophobic surface. This explains why PA can be 'nicked' *in vitro* at the furin site without dissociating into two fragments¹⁴. The remainder of domain 1, which we call domain 1' (residues 168–258), forms the N terminus of the active 63K fragment, PA₆₃.

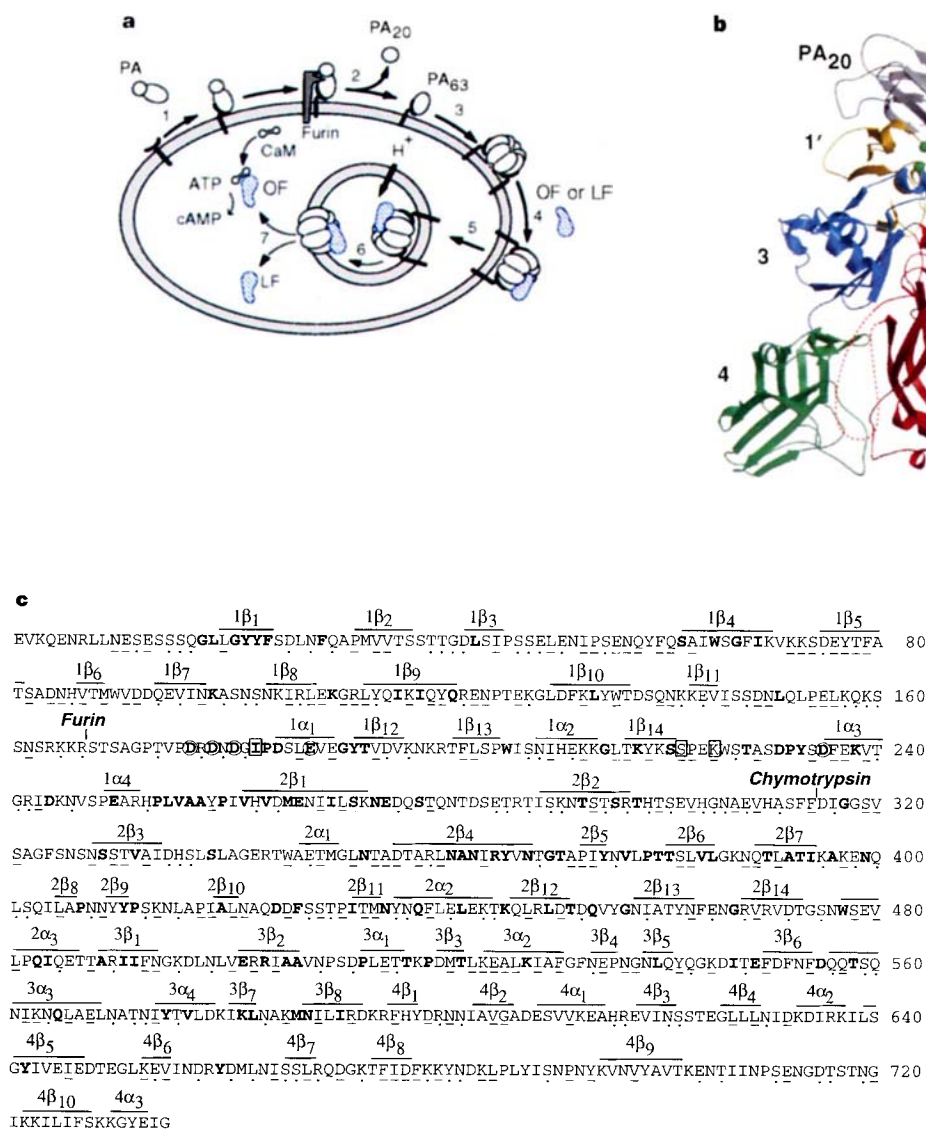
Loss of PA₂₀ leads to heptamer formation by PA₆₃ (ref. 4). The heptamer is water soluble at neutral or basic pH, and inserts into membranes at acidic pH, forming cation-selective channels in both artificial lipid bilayers¹⁵ and cells³. The water-soluble heptamer forms a hollow ring 160 Å in diameter and 85 Å high (Fig. 3). The central lumen has an average diameter of ~ 35 Å, narrowing to ~ 20 Å in some places; it is polar and negatively charged (Fig. 3d),

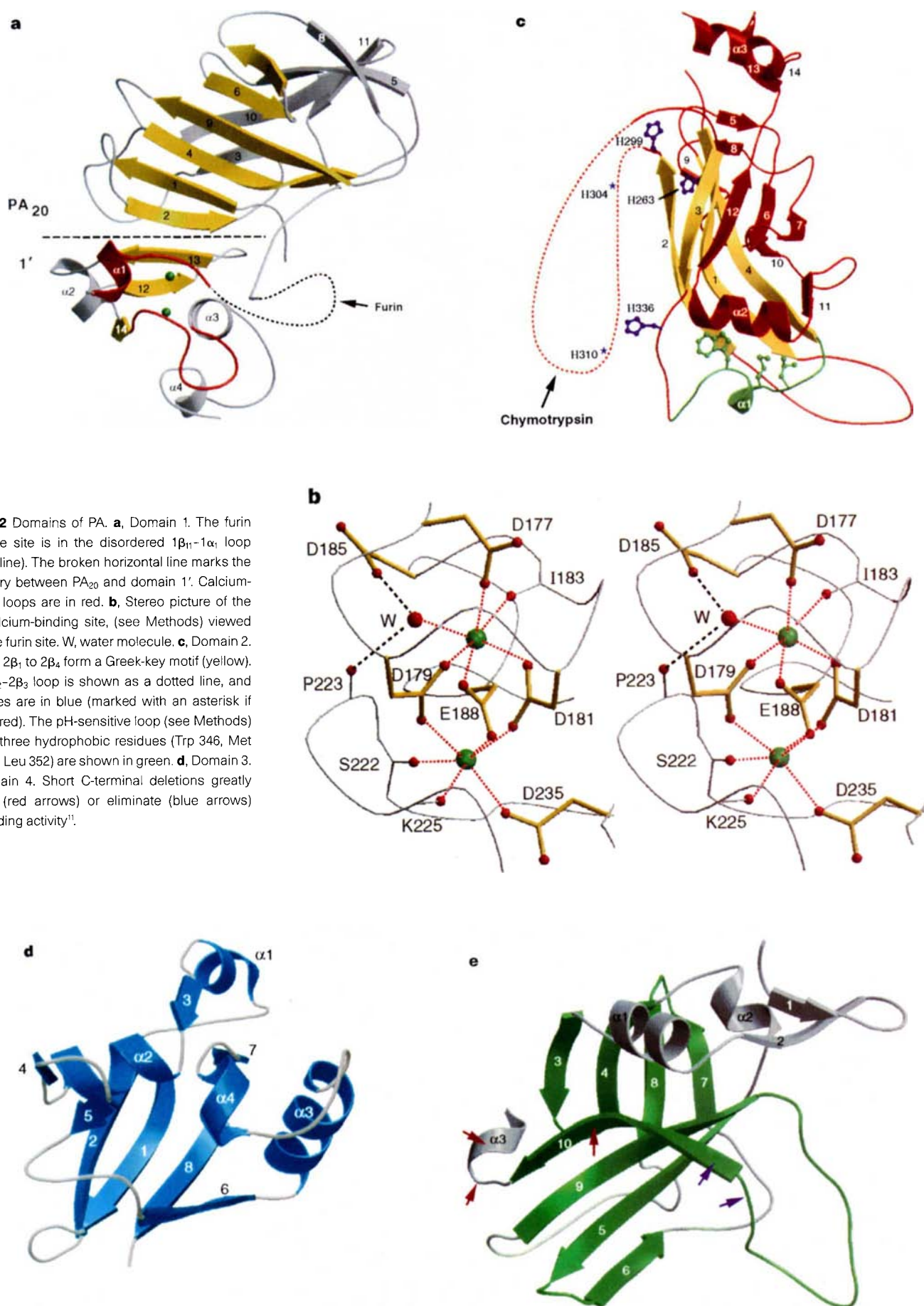
consistent with the cation selectivity of PA₆₃ channels, but also includes a conserved phenylalanine (Phe 427) in a solvent-exposed loop. Monomers pack like pie wedges, with domains 1' and 2 on the inside of the ring and domains 3 and 4 on the outside. Heptamer formation entails no major conformational changes in the monomers, and buries an extensive surface (2,200 Å² per monomer) composed primarily of charged or polar residues from domain 2 and domain 1'. The buried surface is fully accessible in the monomeric PA structure, but substituting full-length PA for PA₆₃ in the heptamer results in a severe overlap among the seven PA₂₀ moieties, showing that heptamerization before furin cleavage is sterically blocked. Residues in the interface are highly conserved, suggesting that the homologous toxins also oligomerize.

The tertiary structure of domain 1' remains largely unchanged on heptamer formation, despite rupture of the β-sheet. The largest movement occurs in the 1β₁₂–1β₁₃ hairpin, which tilts upwards (by 5 Å at its tip) to avoid steric clashes with its neighbour. The domain is stabilized by an extensive interface with domain 2 and internally by the two calcium ions, which link the new N terminus to residues in the core of domain 1' and prevent its becoming disordered. The new hydrophobic surface on domain 1' is completely exposed, forming part of a large, flat, hydrophobic patch on the 'top' of the heptamer (Fig. 3c). This surface provides an obvious site for binding

oedema factor and lethal factor, which bind PA₆₃ with high affinity^{2,16} but cannot bind full-length PA. Monoclonal antibody studies also implicate domain 1' (and the adjacent domain 3) in oedema/lethal factor binding¹⁰.

Because lethal factor binds cells preincubated with PA₆₃ and blocks the ion conductance of preformed PA₆₃ membrane channels¹⁶, the membrane-bound heptamer is likely to be oriented with domain 1' exposed to the extracellular environment, available to bind oedema factor and lethal factor, and with domain 4 and the bottom of domain 2 next to the membrane. How does the heptamer insert into membranes? Our heptamer structure lacks an obvious belt of hydrophobic residues, but proteolysis and mutagenesis experiments have implicated the large 2β₂–2β₃ loop of domain 2 (residues 302–325, a chymotrypsin-sensitive loop with two Phe residues at its tip) in the intoxication process^{11,17}. Electrophysiological measurements on the membrane-inserted heptamer involving point mutations in this loop place residues 304, 306 and 308 inside the channel lumen (E. L. Benson, P. D. Huynh, A. Finkelstein and R.J.C., unpublished data). The loop shows a conserved pattern of alternating hydrophilic and hydrophobic residues, similar to that seen in porins (Fig. 4a) and in the membrane-spanning hairpin of α-haemolysin from *Staphylococcus aureus*¹⁸. In α-haemolysin, each monomer contributes an amphipathic hairpin to a 14-stranded β-





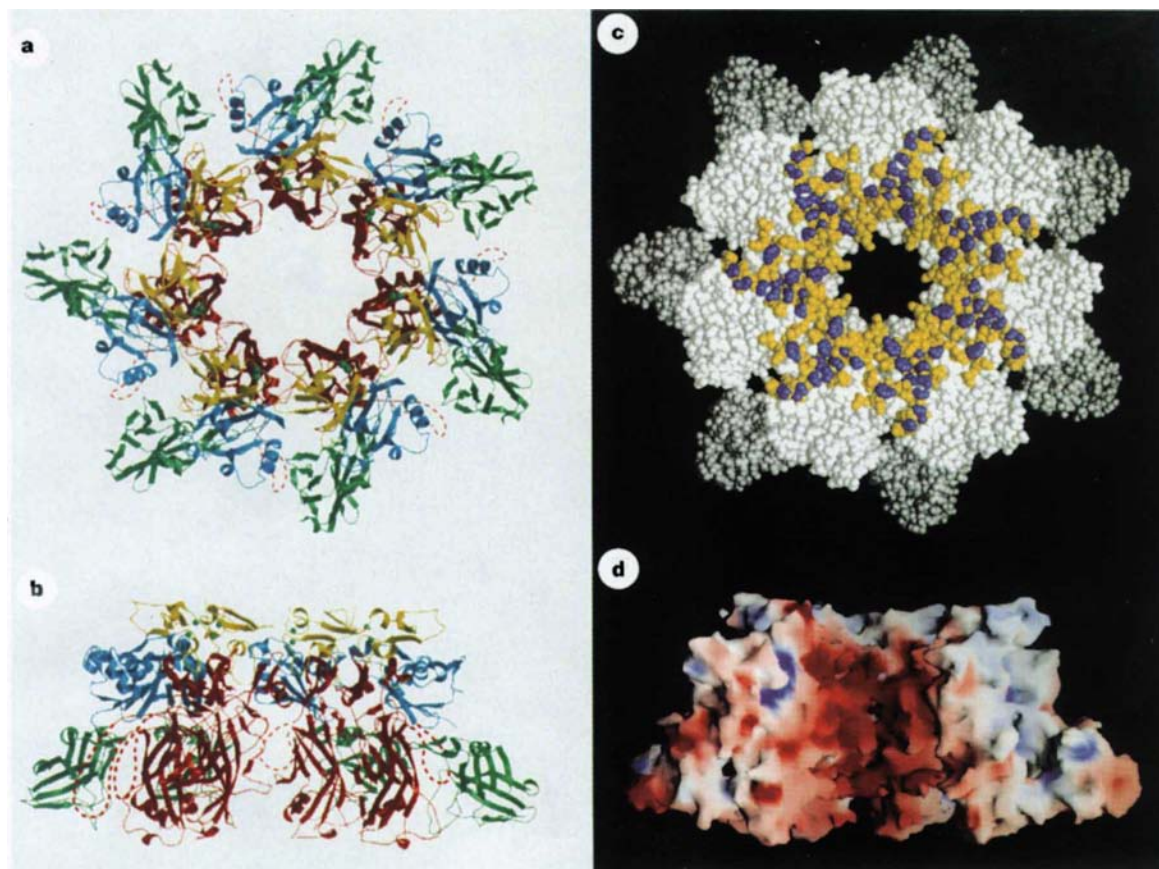


Figure 3 The PA₆₃ heptamer. **a**, Axial view, coloured as in Fig. 1b. **b**, Side view, with three monomers removed to reveal the heptamer interior. **c**, Space-filling model, viewed as in **a**. PA₂₀ removal exposes a large surface area (~1,600 Å² per monomer, yellow and blue), of which 40% is due to hydrophobic side chains (blue). **d**, The heptamer lumen, viewed as in **b**. Regions of positive (blue) and

negative (red) surface potential are indicated. Generated with GRASP²⁹. Homology models of VIP1 and iota-b also have a negatively charged lumen, suggesting that this feature may be important for translocation, an acid-dependent process². The lumen is too small to allow the passage of native folded lethal (or oedema) factor, but large enough to accommodate helices and sub-domains.

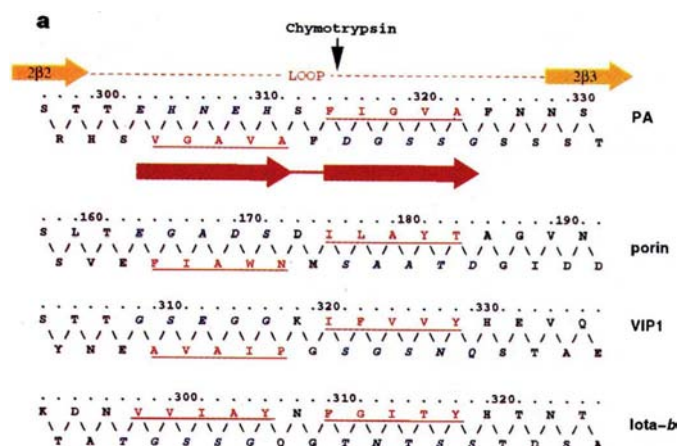


Figure 4 Model of membrane insertion. **a**, Amphipathic hairpin formation by the 2β₂-2β₃ loop of PA and its homologues, with residues forming the hydrophobic face in red and the hydrophilic face in blue. A membrane-spanning hairpin from *Rhodospseudomonas blastic* porin³⁰ is shown for comparison. Cleavage at the chymotrypsin site eliminates toxicity. **b**, Unfolding of the Greek-key motif (strands 2β₁ to 2β₄) to form a β-hairpin extending from the base of domain 2 to the heptamer axis. Histidine positions are shown as blue asterisks. Residues that undergo the order-to-disorder transition in crystals of PA are shown in green. **c**, Association of the seven β-hairpins in the PA₆₃ heptamer to form a 14-stranded, membrane-inserted β-barrel.

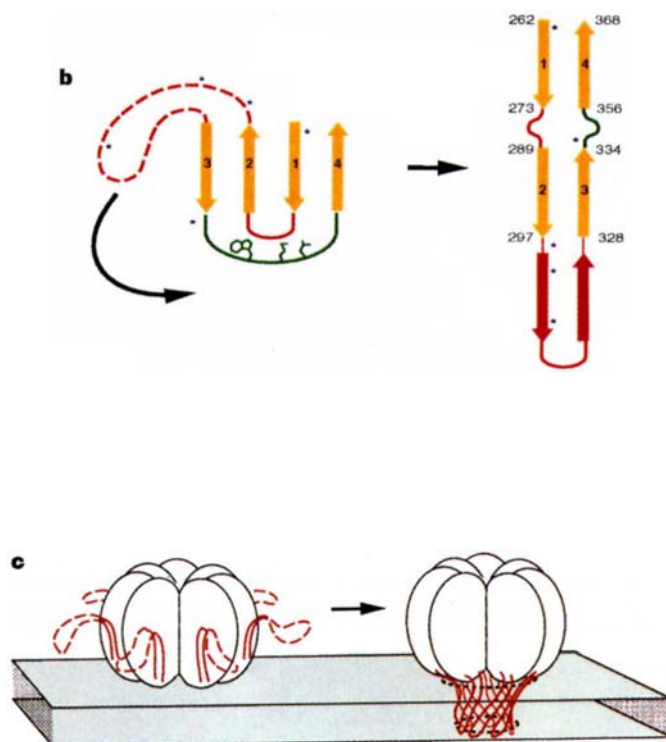


Table 1 Crystallographic statistics

Monomeric PA											
Data set	Nat1a	Nat1b	Nat2	MMN1*	MMN2	MA	K ₂ PtCl ₆	K ₂ PtBr ₆	TTP	U	KI
Resolution (Å)	3.2	2.5	2.1	3.2	3.2	4.0	3.6	3.2	3.6	4.0	3.4
Unique	13,012	28,421	45,183	11,423	13,29	6,686	9,359	12,073	8,180	7,112	10,093
Completeness (%)	90	96	91	85	90	92	96	83	82	94	89
Redundancy	7.0	3.3	3.0	4.2	3.8	2.9	2.4	2.6	3.3	4.0	4.3
R _{sym} (%)†	4.1	6.6	6.7	4.7	9.9	4.5	4.0	7.0	5.1	7.0	7.1
⟨I⟩/⟨σI⟩ in outer shell	4.8	10.7	4.0	3.4	1.5	8.2	6.6	4.8	4.2	3.8	3.5
Sites				5	6	5	2	3	2	4	11
R _{iso} *				8.9	22.3	13.0	11.3	13.9	12.5	11.0	13.1
R _{OUIS} ‡				0.72	0.71	0.86	0.90	0.80	0.81	0.89	0.88
Phasing power				1.1	1.2	0.7	0.6	0.9	0.6	0.5	0.6
Mean overall figure of merit (20–3.2 Å)				0.58							
Refinement statistics:											
	Resolution (Å)		R factor (%)§		R _{free} (%)		R.m.s. bond lengths (Å)¶		R.m.s. bond angles (deg)¶		
crystal form 1	6-2.5		27.1		32.0		0.009		2.0°		
crystal form 2	6-2.1		22.3		29.1		0.006		1.5°		
Heptameric PA ₆₃											
Resolution shell (Å)	9.63	7.64	6.67	6.06	5.63	5.30	5.03	4.81	4.63	4.47	Overall
Unique	3,876	3,924	3,894	3,899	3,856	3,882	3,897	3,894	3,869	2,969	37,959
Completeness (%)	96.5	99.2	99.4	99.4	99.5	99.4	99.7	99.6	99.6	76.6	99.1
Redundancy	2.7	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.1	2.8
⟨I⟩/⟨σI⟩	20.6	16.2	9.9	7.4	5.8	5.1	4.4	4.2	3.9	2.6	9.8
R _{sym} (%)†	3.3	5.0	10.2	14.6	18.9	22.9	26.3	27.7	29.7	32.1	10.2
Statistics of non-crystallographic symmetry phase refinement:											
Averaging R*	19.0	17.7	20.4	24.0	26.6	29.6	31.1	33.5	38.0	49.9	23.9
Correlation☆	0.91	0.91	0.90	0.85	0.83	0.78	0.74	0.71	0.67	0.55	0.87

* Abbreviations for heavy-metal derivatives are defined in the Methods section.

† $R_{\text{sym}} = \sum \sum |I| - I| / \sum I$; ‡ $R_{\text{OUIS}} = \sum |F_{\text{O}} - F_{\text{C}}| / \sum |F_{\text{O}}|$.

§ $R_{\text{factor}} = \sum |F_{\text{O}} - F_{\text{C}}| / \sum |F_{\text{O}}|$ for reflections with $F_{\text{O}} > 2\sigma(F_{\text{O}})$.

|| R_{free} is computed on a randomly selected 10% of the data which were excluded from refinement.

¶ The r.m.s. deviations from ideal values.

* Averaging $R = \sum |F_{\text{O}} - F_{\text{C}}| / \sum |F_{\text{O}}|$.

☆ Correlation coefficient = $\sum_i (F_{\text{O}} - |F_{\text{O}}|)(F_{\text{C}} - |F_{\text{C}}|) / (\sum_i (F_{\text{O}} - |F_{\text{O}}|)^2 - (\sum_i (F_{\text{C}} - |F_{\text{C}}|)^2)^{1/2}$.

barrel, and we propose that PA inserts into membranes in an analogous fashion. The diameter of PA₆₃ membrane channels determined by solute exclusion (~12 Å)¹⁹ is consistent with the size of such a barrel.

The 2β₂–2β₃ loops project outwards from the side of the water-soluble heptamer (Fig. 3). Their assembly into a β-barrel on the heptamer axis could be accomplished most simply if the Greek-key motif formed by the first four strands of domain 2 (2β₁ to 2β₄) were to unfold, with strands 2β₂ and 2β₃ peeling away from the edge of the domain (Figs 4 and 2c). This would involve breaking about as many hydrogen bonds as are created on barrel formation, and is analogous to the conformational change proposed for the pH-induced conversion of transthyretin from its native to its amyloidogenic form²⁰. *In vivo*, the trigger for membrane insertion is provided by acidification of the endosome. *In vitro*, channel formation by PA₆₃ in planar lipid bilayers is accelerated rapidly when the pH is lowered from 7.4 to 6.5 (refs 15, 21), implicating the titration of histidines. Of the nine histidines in PA₆₃, five are contained within the Greek-key motif of domain 2 (Fig. 2c and 4b). Two histidines (His 304 and His 310) lie within the 2β₂–2β₃ loop. Two others (His 263 and His 299) form part of an unusual cluster of buried polar residues (including three arginines (Arg 297, Arg 490 and Arg 504) and one glutamate (Glu 502)) located at the top of the Greek key where the 2β₂–2β₃ loop joins the body of domain 2. Local instability induced by protonation of these histidines would be relieved if strands 2β₂ and 2β₃ peeled away to expose the charged residues to solvent. The fifth histidine (His 336) lies at the bottom of the Greek key in the loop connecting strands 2β₃ and 2β₄. Residues in this loop are ordered in crystals of PA grown at pH 7.5, but are disordered in the crystal form grown at pH 6.0, exposing three hydrophobic residues to solvent. This order-to-disorder

transition may resemble the initial conformational changes that occur in the heptamer during membrane insertion. □

Methods

Structure determination of monomeric PA. (See Table 1.) Crystal form 1 grows in 14–18% PEG 1000, 10% glycerol and 50 mM HEPES, pH 7.5, at 4 °C, and adopts space group P2₁2₁2₁ with $a = 119.8$ Å, $b = 73.8$ Å, $c = 95.0$ Å. Crystal form 2 grows in 12–18% PEG 18K and 50 mM citrate, pH 6.0, at 4 °C, and belongs to space group P2₁2₁2₁ with $a = 99.3$ Å, $b = 93.7$ Å, $c = 82.0$ Å. All data sets listed in Table 1 except Nat2 are from crystal form 1. Three native data sets were used: Nat1a for the multiple isomorphous replacement (MIR) phasing, and Nat1b and Nat2 for refinement. Nat1b was collected at 4 °C at SSRL beamline 7-1 ($\lambda = 1.08$ Å) on a MAR image plate, while Nat2 was collected at –170 °C using 30% PEG 200 as the cryoprotectant on a CCD area detector at CHESS beamline A-1 ($\lambda = 0.92$ Å). Nat1b and Nat2 were processed with DENZO and SCALEPACK²². All other data sets were collected at 4 °C on a Xuong-Hamlin Multiwire area detector using Cu Kα radiation, and were processed with UCSD software²³. Crystal form 1 was solved by MIR. Soaking conditions for heavy-metal derivatives were as follows: MMN1 and MMN2, methylmercuric nitrate at 1 mM for 5 h and 10 mM for 1 h, respectively; MA, 10 weeks in 10 mM mersalyl acid; K₂PtCl₆, 3 days at 10 mM; K₂PtBr₆, 3 days at 2 mM and 3 days at 4 mM; TTP, 2 days in 5 mM dipotassium tetrakis(thiocyanato)platinate (II); UO₂(NO₃)₂, 0.1–1 mM over 4 days; KI₃, 6 days in 10 mM KI₃. Heavy-atom positions were determined by Patterson and cross-Fourier methods, and atomic parameters were refined using MLPHARE²⁴. An MIR map calculated at 3.5 Å showed a clear solvent boundary and several elements of secondary structure. An initial model consisting of ~300 glycine and alanine residues was built using FRODO²⁵ and refined at 2.5 Å resolution with XPLOR²⁶. Recombining model phases with the experimental phases led to an improved map and allowed additional residues to be modelled. The initial sequence assignment was facilitated by iodinating crystals to specifically label

tyrosines and by mercurating crystals of two point mutants, Thr → Cys at 357 and Ser → Cys at 623. Iterative cycles of phase recombination, manual model building and XPLOR refinement led to a nearly complete model, but several ambiguities persisted. These were resolved using crystal form 2, whose structure was solved by molecular replacement and refined at 2.1 Å resolution. Two strong peaks of density in an $F_o - F_c$ map were identified as calcium ions on the basis of coordination geometry, bond distances, and the prevalence of acidic ligands. The presence of calcium was confirmed by atomic absorption spectroscopy (T. Koehler and R.J.C., unpublished data). The two calcium ions are 4.3 Å apart and are bridged by three carboxylate groups. Ca1 (top calcium ion in Fig. 2b) is in a calcium-binding loop like that in the canonical EF-hand²⁷, coordinated by carboxylate oxygens of Asp 177, Asp 179, Asp 181 and Glu 188, the carbonyl oxygen of Ile 183, and a water molecule. Ca2 (lower calcium ion in Fig. 2b) is in a novel motif, coordinated by carboxylate oxygens of Asp 179, Asp 181, Glu 188 and Asp 235, and the carbonyl oxygens of Ser 222 and Lys 225. The model for crystal form 2 consists of 660 (of 735) residues, 390 water molecules, and 2 calcium ions. The model for crystal form 1 contains 680 residues, 2 calcium ions and no water molecules, and will be further refined. No density is seen in either crystal form for residues 1–13, 162–176 and 304–319, consistent with the protease sensitivity of these regions¹⁴. Residues 343–350 shown in green in Figs 2c and 4b, including the three hydrophobic residues indicated (Trp 346, Met 350 and Leu 352), are ordered in crystal form 1 (pH 7.5) and in the PA₆₃ heptamer (pH 8.5), but are disordered in crystal form 2 (pH 6.0). Residues 511–516 of domain 3 form helix 3α₁ in crystal form 1 and in the PA₆₃ heptamer, but are disordered in crystal form 2.

Structure determination of heptameric PA₆₃. (See Table 1.) Crystals grow at room temperature in 2–5% PEG 8K, 10 mM CaCl₂ and 50 mM Tris, pH 8.5, and belong to space group $P2_1$, with $a = 142.8$ Å, $b = 148.3$ Å, $c = 164.1$ Å and $\beta = 107.9^\circ$. Data were collected at -170°C using 30% ethylene glycol as a cryoprotectant on a MAR image plate at the ESRF beamline BM14 ($\lambda = 0.945$ Å). A self-rotation function revealed a peak on the $\kappa = 51^\circ$ section that was 68% as high as the origin peak, indicating the presence of one heptamer per asymmetric unit, with the 7-fold axis parallel to the crystallographic 2-fold screw along b . A cross-rotation function calculated using domains 1' to 4 of the monomer structure gave an unambiguous solution at 8.5σ above the mean, and other strong peaks related by non-crystallographic symmetry. A translation search for one monomer gave a clear peak at 9.9σ above the mean, with the highest noise peak at 2.9σ . Clear solutions were also obtained for the other monomers which, when placed relative to the same origin by a combined translation function, gave a centre of mass in good agreement with the position deduced from the native Patterson. The heptamer model showed good packing in the crystal, and was consistent with electron micrographs. Rigid body refinement of the 7 monomers caused a drop in the crystallographic R -factor from 0.48 to 0.41, and a similar drop in R_{free} (calculated using thin shells of reflections comprising 5% of unique measurements). An additional drop in R_{free} was obtained by refining the four domains of each monomer as independent rigid bodies. The resulting coordinates were used to define the non-crystallographic symmetry operators. Cycles of 7-fold averaging were performed with RAVE²⁸, starting from a set of random phases and a mask derived from the atomic coordinates. The averaging R -factor converged to 23.9% for all measurements from 40 to 4.5 Å, and the real-space correlation coefficients relating the monomers were all 0.90 or higher. The rigid-body refined model showed good fit to the averaged map, which showed well-resolved backbone density but little density for side chains. The strongest features in the map were at 6σ above the mean, and corresponded to the calcium ion sites. Minor conformational changes were readily apparent, including shifts in individual helices in domains 1' and 3 and a shift in the

hairpin formed by residues 194–204. The model was adjusted in these regions, and further rigid-body refinement led to a crystallographic R -factor of 32% and an R_{free} of 36%. The model has not been further refined, owing to a lack of high-resolution data.

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Correspondence and requests for materials should be addressed to C.P. (e-mail: cppl@leicester.ac.uk). Coordinates have been deposited with the Protein Data Bank (1acc).

Finding a gene in a haystack

This edition offers new equipment and reagents to make gene hunting easier, including multiple sequence alignment software, a new DNA electrophoresis system, an *in situ* detection system and a plant DNA extraction system.

TSG-p53 knockout mice

From Taconic

Knockout mice deficient in the p53 tumour suppressor gene as a result of homologous recombination

According to the manufacturer, recent data show the p53 gene is the most commonly mutated gene in human cancers; therefore, the TSG-p53 animal model is useful for research studies concerning p53 gene function, tumour biology and screening potential carcinogens. Heterozygote p53 mice are useful for reduced latency, carcinogenicity screening and are currently being evaluated by the National Toxicology Program. Homozygote p53 mice are used in applications for modulating spontaneous tumour development, such as screening chemopreventive agents. This knockout animal model and other transgenic and knockout rats and mice are now available from the manufacturer.

Reader Enquiry No. 100

Gene analysis

MacVector 6.0

From Oxford Molecular

Multiple sequence alignment with improved firewall compatibility

Oxford Molecular has announced the availability of version 6.0 of MacVector sequence analysis software, which provides advanced, easy-to-use bioinformatics tools and access to on-line services. Of special value to researchers studying sequence relationships (for example, in HIV patient samples), MacVector incorporates a high-performance implementation of Clustal W, a powerful multiple sequence alignment algorithm for protein and DNA sequences. Results from Clustal W are viewed and edited in the new, fully-featured alignment editor. MacVector provides easy access to Entrez sequence databases (compiled from GenBank, EMBL, DDBJ, SWISS-PROT, PIR and others) and supports on-line searches to NCBI (National Center for Biotechnology Information), allowing BLAST searches of sequence databases and retrieval of relevant MEDLINE abstracts. Improved firewall compatibility allows on-line access to be exploited by yet a wider audience. Additionally, MacVector directly provides publication-quality data output of sequences and alignments, without further manipulation, for incorporation into reports and scientific papers.

Reader Enquiry No. 101



The Lightcycler PCR machine from Bio/Gene and Idaho Technology for faster PCR protocols.

Lightcycler PCR machine

From Bio/Gene and Idaho Technology

A PCR machine that is said to perform standard PCR in as little as 15 min

The Lightcycler PCR machine provides valuable information on reaction kinetics, monitoring amplification in real time using fluorescence. It should provide users with the ability to monitor DNA amplification in real time. Users may also see benefits to nucleic acid-based research, specifically concerning the validity and accuracy of measurements made using PCR-based analysis. The Lightcycler combines a micro-volume fluorimeter integrated with a thermal cycler. A range of optical systems is available, producing a versatile machine capable of a variety of types of analysis as alternatives to gel electrophoresis. It is hoped this technology will lead to the development of more rapid assays in a wide variety of sectors.

Reader Enquiry No. 102

Antisense functional genomics

From Sequitur

Second-generation, sequence-based antisense compounds for research, diagnostic and therapeutic applications

Antisense technology can determine gene function and validate small-molecule targets by specifically inhibiting gene expression in cell culture. In addition to target validation studies, this programme can assist in the development of a proprietary position on therapeutic applications of antisense. By providing a complete antisense technology programme, the company allows the client to concentrate on the bio-

logical and clinical aspects of the research. Furthermore, the company does not retain the rights to research findings derived through its antisense programme. The programme includes consulting, proprietary antisense compounds and cellular delivery compounds, computerized target-site selection and detailed protocols for antisense research. The company's techniques have successfully inhibited targeted genes a stated 75–95 per cent of the time by screening only a handful of antisense sites for each target gene. Advantages of this programme are said to include improved specificity and reduced toxicity, target gene inhibition with only three to six compounds screened, and rapid, cost-effective results.

Reader Enquiry No. 103

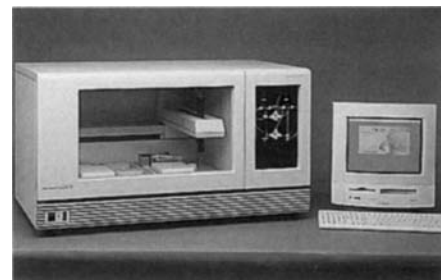
ABI Prism 877 integrated thermal cycler

From PE Applied Biosystems

A new automated workstation for genetic analysis research

Complementing the PE Applied Biosystems DNA sequencing and analysis are the company's instrumentation, reagents and data products. Integration is the key feature of the 877 system; the work surface integrates a 384-well thermal cycler with a high-precision robot capable of pipetting extremely low volumes necessary for both PCR and sequencing reactions, which can now be carried out on a single platform. The system also provides flexibility to choose preprogrammed or customized protocols to be run. The system's complete automation should result in high throughput for a variety of applications. According to the manufacturer, the 877 system can run thousands of PCR and DNA sequencing samples a day, even running unattended overnight. Another benefit of automation is the system's improved reproducibility. As protocols are set up using software and carried out by a highly sensitive robotic arm, manual pipetting is eliminated.

Reader Enquiry No. 104



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GenePrint Powerplex fluorescent STR

From Promega

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The system contains the combined loci of the GenePrint Fluorescent STR Multiplex-CSF1PO, TPOX, TH01, vWA (commonly called CTTv) and a new fluorescent quadruplex, the GenePrint GammaSTR fluorescent STR system, containing the loci D16S539, D7S820, D13S317 and D5S818. The CTTv component is labelled with carboxy-tetramethyl rhodamine and the GammaSTR loci are labelled with fluorescein. All of the STR loci are true tetranucleotide repeats — only two length microvariants have been described in the eight STR loci. Both are in the TH01 locus, the common allele 9.3 and the very rare allele 8.3. This single-tube amplification and single-lane detection of eight loci reveals no *n*+1 artefacts. High-throughput analysis is achieved by comparing the amplified sample DNA fragments directly with allelic ladders or with the fluorescent ladder (CXR). In addition, there is the GenePrint sex determination system (amelogenin labelled with tetramethyl rhodamine), which may be co-amplified with the PowerPlex System to provide a combined gender/identity test consisting of nine loci. The GenePrint PowerplexT fluorescent STR system is said to provide combined matching probabilities of one in 1.18×10^8 for Caucasian Americans, one in 2.61×10^8 for African Americans, and one in 1.45×10^8 for Hispanic Americans (achieved by multiplexing eight loci with no additional customer effort). There is said to be no preparation or mixing of primers or buffers required. All components have been subjected to functional performance testing. Amplification of a 1–25-ng DNA template produces consistent and interpretable results so minimal re-testing is required. For tolerance of sample degradation, all amplification products are between 100 bp and 400 bp long.

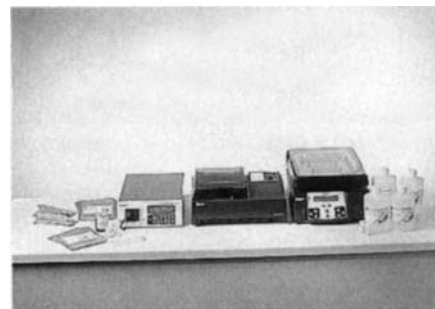
Reader Enquiry No. 105

GenePhor System

From Pharmacia Biotech

Control and convenience in a DNA electrophoresis system

This system consists of the GenePhor electrophoresis unit, GeneGel kits and GeneStain. It offers control, safety and convenience for advanced DNA analysis. By using Peltier temperature regulation on the GenePhor electrophoresis unit, a uniform and stable temperature is achieved around the whole gel, replacing the need for complicated and potentially hazardous water and buffer circulation systems. Temperatures between 5 and 65 °C are reached within a matter of minutes and are easily programmed



GenePhor from Pharmacia Biotech includes a Peltier-cooled electrophoresis unit

using touch-button technology. The electrophoresis unit is used together with precast gels in GeneGel kits and the gels are stained using GeneStain, an automated, preprogrammed unit. This is said to ensure reproducible, high-quality results with convenience and speed. This system should prove to be a useful tool in obtaining results from modern DNA analysis techniques, particularly SSCP, but also VNTR, RAPD, DDRT and microsatellite analysis. The non-denaturing, ready-to-run precast gel from the GeneGel Excel 12.5/24 kit can be loaded with up to 24 samples in the preformed sample wells. Alternatively, the precast gel from the GeneGel Clean 15/24 kit is first rehydrated in the appropriate native or denaturing buffer. The gel is then placed onto the horizontal flatbed of the electrophoresis unit, the preformed buffer strips are mounted, the samples are applied and the lid closed. The temperature is programmed using touch-button controls and the temperature is said to be attained quickly, owing to the Peltier regulation unit. After only two hours of separation, the gel can be placed into GeneStain and, at the press of a button, it automatically performs preprogrammed staining and destaining steps. This utilizes the PlusOne DNA silver staining kit, which includes highly sensitive and non-radioactive staining solutions.

Reader Enquiry No. 106

DiscoverEase

From Genetics

Protein development platform, a functional genomics initiative designed to isolate rapidly and determine functions of critical proteins

The cornerstone of the programme is the company's signal-sequence trap (SST), which rapidly identifies and isolates fragments of genes coding for secreted proteins. To date, this technology has identified a stated 5,000 secreted genes from a wide variety of tissue sources. It should provide researchers with an extensive library of novel human recombinant proteins and full-length genes, along with a corresponding relational database and comprehensive technical assistance. The product is being made available to researchers for biological evaluation.

tion and selection of therapeutic candidates for development and commercialization.

Reader Enquiry No. 107

Insight into hybridization

Gene Frame II

From Advanced Biotechnologies
A gas-tight sealing system for in situ procedures

Designed to improve the reliability of *in situ* hybridization and *in situ* amplification, this system is said to withstand temperatures of up to 97 °C and helps prevent reagent loss due to evaporation. It has been designed for use with standard microscope slides and, depending on the size of sample, double or triple samples can be processed simultaneously. Packaged under cleanroom conditions, Gene Frame II can be autoclaved and sterilized by ultraviolet irradiation. The adhesives on either side of the frame are of differing strengths. The side that adheres to the glass slide has an easy-release adhesive, whereas the reverse side that sticks to the coverslip forms a stronger bond. This means that the coverslip and the frame are removed simultaneously, with no sticky residue left on the slide. The reaction chamber volumes of the frames are increased compared to the original Gene Frame and now have a variety of capacities, including 25 µl, 65 µl and 125 µl.

Reader Enquiry No. 108

GenPoint for biotinylated probes

From Dako

An in situ hybridization detection system for visualizing small quantities of target DNA without the need for costly instrumentation

Based on the catalysed signal amplification (CSA) methodology, GenPoint for biotinylated probes is said to offer high sensitivity and a convenient procedure, with none of the complexities and contamination concerns associated with target amplification methods, such as PCR. For example, Dako says single copies of HPV16 in SiHa cells can be detected in just five hours using this system. The system can also be used with routinely processed tissue (including formalin-fixed paraffin sections) or cell preparations. A similar CSA system is available for immuno-



Dako's GenePoint for biotinylated probes

histochemistry, offering signal amplification 20 to 200 times greater than traditional avidin-biotin methods.

Reader Enquiry No. 109

Oligo processing and analysis

Molecular weight marker XVI and XVII

From Boehringer Mannheim

Two new markers introduced for nucleic acid fragmentation and mapping

According to the manufacturer, brighter banding and higher accuracy are among the benefits of these two new molecular weight markers. The markers are offered for use in the accurate molecular weight determination of double-stranded DNA fragments. Both markers have several features that make them useful for use in nucleic acid fragmentation and mapping. Specific fragments are stated to be three times brighter, offering better orientation and making it easier for researchers to differentiate the bands. For the first time the company is including the quantity in nanograms of each band to facilitate the quantification of DNA fragments generated by PCR or restriction digests separated on agarose gels. Separation of DNA molecular weight marker XVI (250 base pairs) reveals a ladder pattern of twelve double-stranded DNA fragments covering the size range of 250–3,000 bp. DNA molecular weight marker XVII (500 bp) shows a ladder pattern of ten double-stranded DNA fragments covering the size range of 500–5,000 bp.

Reader Enquiry No. 110

Oligo Couple-It

From CPG

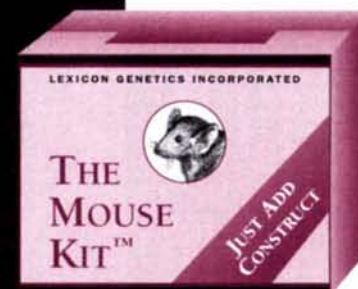
Convenient coupling of primary amine-labelled oligonucleotides and other primary amine-labelled nucleic acids to a magnetic solid support

Using magnetic porous glass (MPG), the kit contains all of the reagents and the magnetic solid support needed to carry out two efficient coupling reactions (10 mg of solid support per reaction). The resulting product is an oligo-coupled magnetic solid support, which can be stored for future use, and is also reusable. The coupled product can be used in solid-phase, nucleic acid-based applications, including solid-phase RT-PCR, subtractive hybridization, purification of specific messages or RNA species, isolation of specific DNA molecules, hybridization-based assays and purification and/or identification of nucleic acid binding proteins. Coupling with the magnetic solid support further enhances the efficiency and convenience of downstream applications by enabling rapid, single-tube assays and in most cases eliminating the need for centrifugation and column chromatography.

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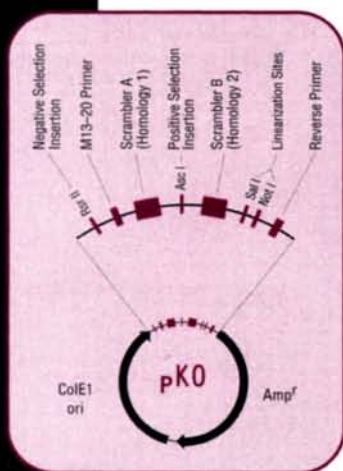
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This is a bench-top instrument designed for automatic cleavage, deprotection and concentration of oligonucleotides following synthesis. Capable of working in conjunction with a variety of DNA synthesizers, it is designed to hold and process up to 12 samples simultaneously. A specially designed cap assembly helps ensure each sample remains isolated and free from contamination. During cleavage and deprotection (typically at 55 °C), the cap assemblies stay closed; during the concentration stage, the valve in the cap opens due to the centrifugal force and allows vacuum concentration to proceed. For added flexibility, incubation and concentration times are programmable, and a post-trap assembly provides odour-free ammonia trapping, neutralization and disposal. Previously, this was only available with one set temperature (55 °C), but now the system can be provided with two user-definable temperature settings for compatibility with different new, fast chemistries.

Reader Enquiry No. 112

N-Linker-CX phosphoramidite

From BioGenex

Designed for multiple labelling of oligonucleotides with amino groups

The new N-Linker-CX phosphoramidite is designed for ease of use, providing more efficient oligonucleotide labelling with amines. The product contains a spacer arm with a cyclohexyl ring that effectively improves the rigidity of the molecule, thus preventing steric hindrance of the bulky dimethoxytrityl group with the oligonucleotide chain. The result is high coupling efficiency (greater than or equal to 99 per cent) for multiple additions and for maximal yield of the oligonucleotide. The linker can be used to synthesize oligonucleotides with multiple amino groups at any position. It is suitable for post-synthesis of oligonucleotides with reporter groups such as biotin, fluorescein and enzymes. Packaged for rapid attachment to most DNA synthesizers, it is used like conventional DNA phosphoramidite monomers. The manufacturer supplies the product as a dry powder for dissolution with anhydrous acetonitrile, and is available in 0.1-mmol, 0.25-g and 0.5-g quantities.

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Nucleix Plus

From Amersham

A new mini-catalogue featuring high-quality nucleotides and nucleosides

Included in the mini-catalogue are full details on non-radioactive dNTPs, which are



Nucleix non-radioactive nucleotides and nucleosides from Amersham.

functionally tested in sequencing and long PCR, as well as premixed dNTP blends (2 mM, 10 mM and 25 mM), which have been optimized for long PCR. Also included are non-radioactive ddNTPs and other modified nucleotides, such as α -thio dNTPs, 2'-modified dNTPs and deoxynucleosides, as well as 3'-modified and other modified dNTPs. Full ordering information is provided for each nucleotide along with a molecular diagram and details of typical applications. Selected technical references are also included in the mini-catalogue.

Reader Enquiry No. 114

Miscellaneous products

RiboQuant

From PharMingen

Multi-probe template sets help in studying role apoptosis in the development and homeostasis of multicellular organisms

Apoptosis plays an essential role for the development and homeostasis of multicellular organisms. This ribonuclease protection assay system employs a series of antisense RNA templates (multi-probe sets) to quantify transcription levels of target genes. The company has released a new panel of human apoptosis template sets for use with the system. These probe sets have been developed to quantify transcriptional levels of genes involved in all aspects of apoptosis, including members of the caspase and bcl2 family, fas ligand receptors and interacting molecules, as well as many others. New template sets include hAPO-1 (FLICE, granzyme B, Mch2, ICERel-111, ICHs, Mch3, ICE, ICHL and ICELAP6), hAPO-2 (bclXL, bclXS, bfl, bad, bik, teak, bax, bcl2 and mcl1), hAPO-3 (FLICE, FASL, FAS, FADD, DR3, FAP, FAF, TRAIL, TNFRp55, TRADD and RIP), hAPO-4 (granzyme A, granzyme B, DAD1, FAST K, Granzyme H, RVP1, Dr-nm23, granzyme 3, Requiem, CAS and perforin) and hAPO-5 (XIAP, TRAF1, TRAF2, CART, NAIP, IAP2, IAP1, TRPM2 and CRAF). Advantages of the system include its high sensitivity and specificity, as well as its capacity to quantify simultaneously many mRNA species in a single sample of total RNA. The assay can be performed on total RNA prepa-

rations derived by standard methods from either frozen tissues or cultured cells without further purification of poly-A⁺ RNA.

Reader Enquiry No. 115

1996/1997 molecular biology catalogue From Genosys Biotechnologies

The catalogue introduces a new range of products for molecular biology research

The Genosys catalogue includes a range of commonly used restriction and modifying enzymes, as well as DNA size markers. It describes specialty products for DNA and RNA isolation, cloning, DNA labelling, recombinant screening and template preparation. For DNA labelling, the E-Link kit contains all the necessary reagents and protocols to label directly up to two oligonucleotide probes with alkaline phosphatase, yielding 0.3–1.5 nmol of labelled oligonucleotide. The kit comes with or without chemiluminescent substrate for non-isotopic detection. It is suited to DNA fingerprinting, northern and Southern blotting and *in situ* hybridization. The company also offers Molt-E-Link, a labelling kit that allows up to ten oligonucleotide probes to be labelled with alkaline phosphatase. The Vectorette II system for amplifying unknown DNA enables genomic walking, intron mapping, promoter localization and sequencing of termini in artificial chromosome clones and cosmids. OligoPlate enables researchers to have custom oligonucleotides covalently attached to 96-well microtitre plates. Its use allows for capturing and analysing PCR products and is amenable to high-throughput processing and automation. Researchers can request one or two oligonucleotides per plate, or a third format that utilizes a universal capture plate, enabling a variety of different capture probes to be used. It is also suited for capturing and detecting PCR products that incorporate biotinylated primers with subsequent colorimetric or chemiluminescent detection.

Reader Enquiry No. 116

Separation and extraction

Vectrex matrices

From Vector Labs

For reversible affinity binding of biotinylated and fucosylated nucleic acids and proteins

Two new matrices are available that allow reversible binding of biotinylated or fucosylated molecules for potential use in probe purification, PCR, cDNA and subtraction library construction, hybridization analysis, and *in vitro* mutagenesis. The matrix is a highly crosslinked sugar polymer with a large surface area for maximum binding of large or small molecules and low non-specific binding of charged molecules. Avidin DLA (a modified form of avidin with a reduced affinity for biotin) has been conju-



gated to the surface of the particle. Biotinylated molecules can be bound to Vectrex Avidin DLA and eluted with biotin under non-denaturing conditions. Alternatively, fucosylated nucleic acids can be bound to the company's AAL (Aleuria aurantia lectin, a fucose-specific lectin). Subsequently, this can be eluted with fucose under non-denaturing conditions. According to Vector, nucleic acids can be easily fucosylated or biotinylated in less than 60 min using the FastTag labelling systems.

Reader Enquiry No. 117

Nucleon PhytoPure

From ScotLab

A system for extracting DNA from plant samples — capable of producing high yields of DNA in a shorter time

This system is designed to be more efficient, as well as considerably simpler to use than conventional methods. Whereas most plant DNA extraction techniques are effective in removing proteins, they can be much less successful with polysaccharides. With polysaccharides being common contaminants in plant DNA extracts, the result is often difficult-to-handle, 'slimy' DNA pellets. This problem is compounded as polysaccharides, particularly those of an anionic nature, can be inhibitory to the further enzymatic analysis of the DNA. The Nucleon PhytoPure DNA extraction system has been developed specifically to solve these problems. The system protocol is rapid and eliminates the requirement for phenol or CTAB (cyclohexadecyltrimethyl ammonium bromide). After breaking of

the cell wall, the cells are lysed in a solution containing potassium SDS, which is known to form complexes with proteins and polysaccharides. Chloroform is then added along with the specially modified Nucleon PhytoPure silica suspension. This silica suspension covalently binds polysaccharides, resulting in a high-quality DNA preparation at the end of the protocol. The silica particles contain free boric acid groups, which are reactive with polysaccharides, thereby removing them from the sample. Furthermore, during extraction this product forms a semi-solid stratum between a lower organic layer and the upper DNA-containing aqueous phase. This facilitates DNA recovery without repeating partitions, ensuring high yields of high-quality DNA suitable for further analysis by restriction enzyme digestion, RAPD and AFLP.

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